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HIGH DENSITY ARRAYS FOR PROTEOME ANALYSIS AND METHODS AND COMPOSITIONS THEREFOR

Abstract:

The present invention provides high-density arrays comprising a primary protein array and a secondary antibody array, wherein the secondary array comprises monoclonal antibodies and/or antibody variants or derivatives that bind specifically or non-specifically to one or more proteins in the primary array, and wherein the secondary array is used to determine the protein profile of a cell, tissue, organ or whole organism or a cellular extract, lysate or protein fraction derived ther
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(54) Title: HIGH DENSITY ARRAYS FOR PROTEOME ANALYSIS AND METHODS AND COMPOSITIONS THEREFOR

(57) Abstract

The present invention provides high-density arrays comprising a primary protein array and a secondary antibody array, wherein the secondary array comprises monoclonal antibodies and/or antibody variants or derivatives that bind specifically or non-specifically to one or more proteins in the primary array, and wherein the secondary array is used to determine the protein profile of a cell, tissue, organ or whole organism or a cellular extract, lysate or protein fraction derived therefrom. Also provided are methods of determining the epitope profile of cells, tissues, organs and whole organisms and cellular extracts, lysates or protein fractions derived therefrom, using the high density protein arrays of the invention, in particular in relation to diagnostic and therapeutic applications. The invention further provides for the enrichment of native proteins from complex mixtures of cellular proteins by employing one or more antibodies uniquely recognising an antigen of interest as defined by recognition patterns obtained when screening secondary antibody arrays against primary antigen arrays. In addition, one or more antibodies can be employed to produce a unique tag for target antigens and is employed to follow the expression levels of complex mixtures of cellular proteins and is conducted independently of the separation sciences. A similar approach is employed to produce a fingerprint of a biological sample, based upon recognition of a multiplicity of individual antigens providing a pattern useful in recognition or diagnosis of a group of biological samples of interest in healthy and diseased samples, or test and control experimental situations for diagnostic purposes.

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HIGH DENSITY ARRAYS FOR PROTEOME ANALYSIS AND METHODS AND COMPOSITIONS THEREFOR

FIELD OF THE INVENTION

The present invention relates generally to high density protein arrays for proteome analysis. More particularly, the present invention provides high-density arrays comprising a primary protein array and a secondary antibody array, wherein the secondary array comprises monoclonal antibodies and/or antibody variants or derivatives that bind specifically or non-specifically to one or more proteins in the primary array, and wherein the latter information allows the secondary array to be used to determine the protein profile of the expression levels of a multiplicity of individual proteins in parallel, from biological extracts derived from a cell, tissue, organ or whole organism or a cellular extract, lysate or protein fraction derived therefrom. Preferably, the profile that is obtained for any biological sample using the high-density arrays of the present invention comprises a sub-set of the total antigenic diversity of said biological sample that is also immunologically cross-reactive with one or more of the proteins in the primary protein array. The present invention also relates generally to methods of determining the epitope profile of cells, tissues, organs and whole organisms and cellular extracts, lysates or protein fractions derived therefrom, using the high density protein arrays described herein, in particular in relation to diagnostic and therapeutic applications.

GENERAL

Bibliographic details of the publications referred to in this specification are collected at the end of the description.

As used herein the term "derived from" shall be taken to indicate that a specified integer may be obtained from a particular source albeit not necessarily directly from that source.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated step or element or integer or group of steps or elements or integers but not the exclusion of any other step or element or integer or group of elements or integers.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

BACKGROUND TO THE INVENTION

High density arrays of nucleic acids, such as cDNA's and synthetic oligonucleotides, have revolutionised the way cellular activities within living systems are assayed, because these systems allow for a high degree of automation, repetitive analysis, and duplication of the array at minimal cost (*see* review by Fraser and Fleischmann, 1997). Numerous pharmaceutical companies have now applied nucleic acid hybridisation assays to very large gridded arrays of cDNA's (eg. the IMAGE consortium cDNA's, available from Research Genetics), and more recently, these technologies have become compatible with silicon chip technology, which offers considerable advantages in terms of miniaturisation of the arrays (Goffeau, 1997). Moreover, such systems posses the advantage of affording the potential to screen large numbers of individuals

or populations for differences between healthy and diseased tissues and to monitor the effects of ageing, a variety of stresses, drug administrations, developmental / cell cycle, infection, disease, etc.

Notwithstanding the advantages conferred by nucleic acid-based arrays, such systems provide limited information on the cellular processes or disease associations in living organisms because, with the possible exception of DNA/protein interactions that regulate gene expression, a knowledge of protein expression, rather than nucleic acid expression, is of greatest utility in analysing cellular processes. Protein profiles of cells, tissues, organs or whole organisms provide important information on protein synthesis and turnover and a true indication of the health or otherwise of a cell, tissue, organ or organism. The phenotype of living cells is dependent upon the protein gene-products produced therein at any given time or developmental stage.

Protein-based approaches for analysing cellular processes have been reviewed by Humphery-Smith and Blackstock (1997) and Humphery-Smith *et al.*, (1997). The technology is at present limited by several factors, including an absolute reliance upon conventional protein separation technologies, such as 2-dimensional gel electrophoresis, chromatographic procedures (including the more rapid technologies of FPLC and SMART, supplied by Pharmacia, Uppsala, Sweden), capillary electrophoretic techniques and mass spectrometry, to separate mixtures of proteins into individual analytes and/or to enrich individual analytes, a lack of sensitivity which prevents the detection of entire proteomes (i.e. the potential protein output of a cell, tissue, organ or organism), and an inability to analyse entire proteomes, rather than merely individual proteins, in a single assay.

In particular, most conventional protein separation technologies are expensive, highly technically-demanding and require a trial-and-error approach for the separation and/or enrichment of individual proteins.

Moreover, the temporally-specific and developmentally-specific nature of gene expression means that not all proteins are produced at all times under all physiological conditions, nor in all cells or tissues within a given organism. Thus, a reliance upon single cellular extracts of proteins derived from a specific developmental stage of an organism, or alternatively, derived from a specific time-point, has not facilitated the routine analysis of the entire proteome of a cell, tissue, organ or organism.

The direct transfer of technologies used in nucleic acid-based screening approaches to protein-based screening approaches is not possible, because of the fundamentally different nature of nucleic acid and protein. For example, the advantages conferred by the use of polymerase chain reaction (PCR) in nucleic acid-based approaches are irrelevant to protein-based approaches, because that technology does not result in the amplification of protein and there is no equivalent means for the amplification of proteomes, or even mixtures of proteins, *in vitro*. Additionally, there is no means by which *any* protein may be produced *in vitro* without the use of a structural gene template or an expression gene constructs comprising same, and genetic manipulations to produce high-density arrays of expression gene construct matrices that are representative of the entire proteome of a cell, tissue, organ or organism, are not possible.

In work leading up to the present invention, the inventors sought to develop multiple array screening systems that could be readily applied to the analysis of entire proteomes. In particular, the inventor has developed a high density array comprising a primary and secondary array, and a screening system for use therewith, to provide unique tags in the secondary array, such as one or more animal-derived or phage-derived monoclonal antibodies, to each of the elements contained within a primary high-density array of proteins. The high density array developed by the inventor provides the means by which at least one tag is obtained per protein of the near-to-total proteome of *any* organism. Although many antibodies of the secondary array will be highly non-specific, a combination of one or many antibodies, for example hundreds

of antibodies, can engender a unique response, and the non-specific responses can be used to validate the experimental procedure as internal controls, or alternatively or in addition, to provide tags to different regions of the same protein molecule.

SUMMARY OF THE INVENTION

The present invention provides a method of substantially determining the proteome content of cells, tissues and organisms or fractions of any of the foregoing by the utilisation of a first or primary high density protein array and a secondary high density antibody array.

Accordingly, one aspect of the invention provides a method of determining the protein profile of a biological sample comprising:

(i) preparing a primary array of proteins $a^1_{(Xn,Yn)}$, $a^2_{(Xn,Yn)}$, $a^3_{(Xn,Yn)}$,.....,$a^n_{(Xn,Yn)}$ comprising a multiplicity of protein elements designed to emulate a significant portion of the antigenic diversity of a cell, tissue, organ, organism from which the biological sample is derived, wherein a^1,a^2,a^3,.....,a^n are proteins; Xn is the coordinate of any particular protein along a first dimension of said array; Yn is the coordinate of any particular protein along a second dimension of said array; and wherein n is any positive finite number;

(ii) screening the primary array with a plurality of monoclonal antibodies and/or antibody variants and/or derivatives one-at-a-time, or a reduced pool thereof one-at-a-time, so as to determine those antibodies and/or antibody variants or derivatives that bind to one or more proteins in said primary array;

(iii) preparing a secondary array of monoclonal antibodies and/or antibody variants or derivatives $Ab^1_{(Xn,Yn)}$, $Ab^2_{(Xn,Yn)}$, $Ab^3_{(Xn,Yn)}$,......,$Ab^n_{(Xn,Yn)}$, wherein Ab^1,Ab^2,Ab^3,.....,Ab^n are monoclonal antibodies and/or antibody variants or derivatives that bind specifically or non-specifically to one or more proteins in the primary array; Xn is the coordinate of any particular monoclonal antibody and/or antibody variant or derivative along a first dimension of said array; Yn is

the coordinate of any particular monoclonal antibody and/or antibody variant or derivative along a second dimension of said array; and wherein n is any positive finite number; and

(iv) screening the secondary array with said biological sample to determine those proteins in said biological sample which bind to one or more monoclonal antibodies and/or antibody variants or derivatives in said secondary array in a uniquely-definable manner.

In a preferred method of the invention, the secondary high density antibody array is derived by means of a polyclonal immune response to a composite soup of the elements employed to generate the individual elements of the primary high density protein array. Preferably the primary high density protein array is an antigen array.

In an alternative embodiment, this aspect of the invention may be used for comparative purposes, to determine whether the protein profile of the "test sample" possesses any differences in terms of expressed proteins, to a biological standard or reference.

Accordingly, a further aspect of the invention provides a method of determining one or more proteins that are differentially-expressed between cells, tissues, organs, or organisms or biological samples derived therefrom comprising:

(i) preparing a primary array of proteins $a^1_{(Xn,Yn)}$, $a^2_{(Xn,Yn)}$, $a^3_{(Xn,Yn)}$,.....,$a^n_{(Xn,Yn)}$ comprising a multiplicity of protein elements designed to emulate a significant portion of the antigenic diversity of a cell, tissue, organ, organism from which the biological sample is derived, wherein a^1,a^2,a^3,....,a^n are proteins; Xn is the coordinate of any particular protein along a first dimension of said array; Yn is the coordinate of any particular protein along a second dimension of said array; and wherein n is any positive finite number;

(ii) screening the primary array with a plurality of monoclonal antibodies and/or antibody variants and/or derivatives one-at-a-time, or a reduced pool thereof one-at-a-time, so as to determine those antibodies and/or antibody

variants or derivatives that bind to one or more proteins in said primary array;

(iii) preparing a secondary array of monoclonal antibodies and/or antibody variants or derivatives $Ab^1_{(Xn,Yn)}$, $Ab^2_{(Xn,Yn)}$, $Ab^3_{(Xn,Yn)}$, ..., $Ab^n_{(Xn,Yn)}$, wherein Ab^1,Ab^2,Ab^3,....,Ab^n are monoclonal antibodies and/or antibody variants or derivatives that bind specifically or non-specifically to one or more proteins in the primary array; Xn is the coordinate of any particular monoclonal antibody and/or antibody variant or derivative along a first dimension of said array; Yn is the coordinate of any particular monoclonal antibody and/or antibody variant or derivative along a second dimension of said array; and wherein n is any positive finite number; and

(iv) separately screening the secondary array with two or more biological samples derived from said cells, tissues, organs, or organisms, and comparing the signals obtained using each of said biological samples to determine those proteins which are differentially expressed.

In both of the above-mentioned embodiments, one or more of the proteins in the primary array which form antigen-antibody complexes with elements of the secondary array as determined at (iv) may also be identified, by determining the coordinates (Xn,Yn) of said protein(s) which bind to the monoclonal antibodies and/or antibody variants or derivatives detected by screening the secondary array.

The present invention clearly extends to any proteins isolated from the biological sample, once such proteins have been identified using the inventive method.

It is also an object of this invention to provide a method of construction of high density screening arrays that are useful for simultaneously detecting a plurality of specific proteins in biological samples.

This invention is predicated on the exploitation of the exquisite sensitivity of antibodies and antibody derivatives, in particular monoclonal antibodies, for the immunocapture

of antigens. Clonal selection, or recombinant molecular methods for the generation of molecular diversity, inherent in antibody production allows for the simultaneously production of antibodies against a multitude of antigens. However, at present, screening technologies are not directed towards dissecting *in parallel* the many monoclonal elements from within a polyclonal response, or are they directed to understanding the totality of cellular protein content. Thus, this invention is reliant upon taking advantage of the natural capacity of the polyclonal immune response to detect more than one antigen following a given immunisation procedure and harnessing this capacity to avoid having to screen one antigen by one monoclonal antibody one at a time. Multiple antibodies arranged in high density arrays can then be used to screen proteins derived from biological samples. In particular, it has been found that the simultaneous action of screening high density antigen arrays simultaneously with monoclonal or phage-derived antibodies, with the intention of characterising antibody specificities and with the intention of creating a secondary antibody array, can be used to detect expression levels of cellular or excreted proteins. The essential use of two high-density arrays (i.e. a primary array and a secondary array), facilitates the analysis of proteomes in a time-effective and cost-effective manner and, in particular provides a time-effective and cost-effective means of obtaining a predetermined knowledge of individual antibody specificity and cross-reactivity in a high-throughput environment. This process allows initial antibody screening and antibody specificity testing to be combined into a single step, with respect to the multiplicity of protein elements contained within the primary array.

Accordingly, a further aspect of the invention provides an array for use in determining the protein profile of a cell, tissue, organ or organism or a biological sample derived therefrom, comprising:

(i) a primary array of proteins $a^1_{(Xn,Yn)}$, $a^2_{(Xn,Yn)}$, $a^3_{(Xn,Yn)}$,.....,$a^n_{(Xn,Yn)}$ comprising a multiplicity of protein elements designed to emulate a significant portion of the antigenic diversity of a cell, tissue, organ, organism from which the biological sample is derived, wherein a^1,a^2,a^3,.....,a^n are proteins; Xn is the coordinate of

any particular protein along a first dimension of said array; Yn is the coordinate of any particular protein along a second dimension of said array; and wherein n is any positive finite number; and

(ii) a secondary array of monoclonal antibodies and/or antibody variants or derivatives $Ab^1_{(Xn,Yn)}$, $Ab^2_{(Xn,Yn)}$, $Ab^3_{(Xn,Yn)}$,.....,$Ab^n_{(Xn,Yn)}$, wherein Ab^1,Ab^2,Ab^3,....,Ab^n are monoclonal antibodies and/or antibody variants or derivatives that bind specifically or non-specifically to one or more proteins in the primary array; Xn is the coordinate of any particular monoclonal antibody and/or antibody variant or derivative along a first dimension of said array; Yn is the coordinate of any particular monoclonal antibody and/or antibody variant or derivative along a second dimension of said array; and wherein n is any positive finite number.

The present invention clearly extends to arrays of monoclonal antibodies or antibody variants or derivatives that are produced by screening the primary protein array described herein. Accordingly, a further aspect of the invention provides an array of monoclonal antibodies or antibody variants or derivatives comprising the antibodies $Ab^1_{(Xn,Yn)}$, $Ab^2_{(Xn,Yn)}$, $Ab^3_{(Xn,Yn)}$,....., $Ab^n_{(Xn,Yn)}$, wherein Ab^1 ,Ab^2 ,Ab^3 ,....,Ab^n are monoclonal antibodies and/or antibody variants or derivatives, Xn is the coordinate of any particular monoclonal antibody and/or antibody variant or derivative along a first dimension of said array, Yn is the coordinate of any particular monoclonal antibody and/or antibody variant or derivative along a second dimension of said array, n is any positive finite number; and wherein said array is produced by a method comprising:

(i) preparing a primary array of proteins $a^1_{(Xn,Yn)}$, $a^2_{(Xn,Yn)}$, $a^3_{(Xn,Yn)}$,.....,$a^n_{(Xn,Yn)}$ comprising a multiplicity of protein elements designed to emulate a significant portion of the antigenic diversity of a cell, tissue, organ, organism from which the biological sample is derived, wherein a^1,a^2,a^3,....,a^n are proteins; Xn is the coordinate of any particular protein along a first dimension of said array; Yn is the coordinate of any particular protein along a second dimension of said array; and wherein n is any positive finite number;

(ii) screening the primary array with a plurality of monoclonal antibodies and/or antibody variants and/or derivatives one-at-a-time, or a reduced pool thereof one-at-a-time, so as to determine those antibodies and/or antibody variants or derivatives that bind to one or more proteins in said primary array;

(iii) preparing said secondary array of monoclonal antibodies and/or antibody variants or derivatives using those monoclonal antibodies and/or antibody variants or derivatives that bind specifically or non-specifically to one or more proteins in the primary array.

Preferably, the secondary antibody array is prepared by selecting monoclonal antibodies or antibody variants that bind to proteins in a primary array which are derived from the same cell-type, tissue-type, organ-type, bodily fluid-type, blood-type, or serum-type as a biological sample which is to be used to screen the array.

It is also an object to overcome the difficulty of fabricating tens of thousands of antibodies without calling upon an equivalent number of antibody-generating animals. Accordingly, in a preferred embodiment of the present invention, one or more of the monoclonal antibodies and/or antibody variants or derivatives are derived from hybridomas or other cells, or alternatively, from recombinant bacteriophage or viruses, which each express antibodies or antibody variants or derivatives which bind to one or more proteins in the primary array. More preferably, the hybridomas or other cells, or recombinant bacteriophage or viruses, each express antibodies or antibody variants or derivatives which bind to proteins in the primary array in a specific or non-specific manner .

Further aspects of the present invention relates to the applications of the inventive secondary antibody array to the diagnosis and prophylactic and therapeutic treatment of humans and other animals for medical conditions. In such embodiments, it is preferred that the secondary antibody array is prepared by selecting monoclonal antibodies or antibody variants that bind to proteins in a primary array which are

derived from a healthy individual that does not exhibit symptoms associated with the medical condition being diagnosed or treated.

Accordingly, a further aspect of the invention provides a method of diagnosing a human or animal subject for a medical condition, ailment, illness or immune response or a predisposition for said medical condition, ailment or illness, said method comprising:

(i) screening the secondary array of the invention with a biological sample derived from said subject comprising a cell, tissue, or organ sample, bodily fluid sample, blood or serum sample, or a fraction, derivative or protein extract of any one or more of said samples; and

(ii) comparing the proteins detected for the biological sample at (i) with the proteins detected for a biological standard derived from a healthy individual, wherein differences between the biological sample the biological standard are indicative of said medical condition, ailment, illness or predisposition.

A still further aspect of the invention provides a method of diagnosing a human or animal subject for a medical condition, ailment, illness or immune response or a predisposition for said medical condition, ailment or illness, said method comprising:

(i) separately screening either or both the primary and/or secondary arrays of the present invention with:

(a) a biological sample derived from said subject comprising a cell, tissue, or organ sample, bodily fluid sample, blood or serum sample, or a fraction, derivative or protein extract of any one or more of said samples; and

(b) a biological standard derived from a healthy individual; and

(ii) comparing the proteins detected for said biological sample with the proteins detected for said biological standard at (i), wherein differences between the biological sample the biological standard are indicative of said medical condition, ailment, illness or predisposition.

According to this aspect of the invention, it is particularly preferred that the biological sample and the biological standard are derived from the same cell-type, tissue-type, organ-type, bodily fluid-type, blood-type, or serum-type.

Preferably, wherein the inventive method is to be used for diagnosing an immune response in a human or animal subject, it is preferred that the biological sample comprises blood or serum or a fraction or derivative of each thereof.

The diagnostic methods described herein clearly extend to applications wherein the biological sample is obtained from the subject prior to screening or alternatively, wherein the is prepared array for screening with the biological sample and/or the biological standard. Such preparation may involve the selection of monoclonal antibodies or antibody variants that bind to proteins in a primary array which are derived from a healthy individual and from the same cell-type, tissue-type, organ-type, bodily fluid-type, blood-type, or serum-type as the biological sample.

A still further aspect of the invention extends to compositions for the therapeutic or prophylactic treatment of a human or other animal subject, said compositions comprising a suite of protein elements and/or responsive antibody elements of relevance to disease genesis and/or disease susceptibility that have been identified by screening the primary and/or secondary array of the present invention and preferably subsequently isolated, in combination with a pharmaceutically-acceptable carrier or diluent.

According to this aspect of the invention, it is particularly preferred that the active ingredient of such compositions is a composite of the multiplicity of elements employed in the construction of the primary array, used in approximately equimolar ratio at a sufficiently-high concentration of each individual protein component to produce an antibody response to each of said protein components, preferably a humoral and/or cellular immune response to each of said protein components, in a subject to which

said composition is administered, rather than merely producing a response to a few proteins in a naturally-occurring cellular soup.

This aspect of the invention further extends to the use of such compositions in therapeutic, diagnostic and intervention protocols or for inclusion in drug screening programmes or molecular characterisation.

Accordingly, a still further aspect of the invention contemplates a method of therapeutic treatment of a human or animal subject for a medical condition, ailment, or illness comprising administering the composition *supra* to said subject for a time and under conditions sufficient for the symptoms of said medical condition, ailment, or illness to abate.

A still further aspect of the invention contemplates a method of prophylactic treatment of a human or animal subject for a predisposition to a medical condition, ailment, or illness comprising administering the composition *supra* to said subject for a time and under conditions sufficient for an antibody response or protective immune response to occur.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation showing the dissection in parallel of the monoclonal elements from within a polyclonal response in animal-based or phage-based expression systems.

Figure 2 is a graphical representation showing the populations of B cells within an immunised mouse (bell-shaped curve) and normally sampled during hybridoma production and screening procedures prior to serial dilution and usually in conjunction with a few mice. In each case, approximately 400-600 viable hybridoma post-fusion events (----) are obtained, as opposed to that population (_____)of pre-clonally-diluted hybridomas which is intended to be sampled per mouse, comprising at least one order

of magnitude more clones per mouse. The efficiency of the antibody production procedure is therefore dependent upon the efficiency of the immunisation protocol with respect to the antigens being presented to the mouse immune system, or the immune system of any other host, as the case may be.

Figure 3-I is a diagrammatic representation showing microtitre plate-sized PVDF or nitrocellulose membranes capable of containing 30,000 distinct antigens for a high throughput western blotting of IPTG-induced expression libraries or for gridding colonies for growth and induction on IPTG-containing agar, with ready-accessible current gridding technologies to array using sets of pins that dip one or more times into expression libraries. The former is preferred to reduce the likelihood of colony smearing or merging during growth.

Figure 3-II is a diagrammatic representation showing stacks of the microtitre plate-sized PVDF or nitrocellulose membranes presented in Figure 3-I.

Figure 4 is a schematic representation showing the total potential antigenic diversity encoded by a given genome (rectangle) and the proportion of said total potential antigenic diversity exposed on a primary array in conjunction with an expression library for that genome (oval). Conformational epitopes and antigens that are simply not cloned as a result of poor gene library quality, or genes that encode toxic proteins or which are unstable when cloned, lie outside the oval field.

Figure 5 is a schematic representation showing the dissection in parallel of the monoclonal elements from within a polyclonal response for the screening of viable B cell hybridoma culture supernatants, based upon a predetermined knowledge of monoclonal antibody specificity.

Figure 6 is a copy of a photographic representation of a dot blot of mouse bleeds against 12 immunising antigens that were used as a mixture to immunise mice.

Antigens were dotted onto nitrocellulose and screened with antisera obtained from each of four immunised mice. The Figure indicates four panels of 12 antigens, each panel corresponding to a different mouse antisera used in the screening. Numbers at the side of each panel indicate the number of the mouse from which antisera were obtained. Numbers within each panel indicate the antigen (Table 1)

DETAILED DESCRIPTION OF THE INVENTION

One aspect of the present invention provides an array for use in determining the protein profile of a cell, tissue, organ or organism or a biological sample derived therefrom, comprising:

(i) a primary array of proteins $a^1_{(Xn,Yn)}$, $a^2_{(Xn,Yn)}$, $a^3_{(Xn,Yn)}$,.....,$a^n_{(Xn,Yn)}$ comprising a multiplicity of protein elements designed to emulate a significant portion of the antigenic diversity of a cell, tissue, organ, organism from which the biological sample is derived, wherein a^1,a^2,a^3,....,a^n are proteins; Xn is the coordinate of any particular protein along a first dimension of said array; Yn is the coordinate of any particular protein along a second dimension of said array; and wherein n is any positive finite number; and

(ii) a secondary array of monoclonal antibodies and/or antibody variants or derivatives $Ab^1_{(Xn,Yn)}$, $Ab^2_{(Xn,Yn)}$, $Ab^3_{(Xn,Yn)}$,.....,$Ab^n_{(Xn,Yn)}$, wherein Ab^1,Ab^2,Ab^3,....,Ab^n are monoclonal antibodies and/or antibody variants or derivatives that bind specifically or non-specifically to one or more proteins in the primary array; Xn is the coordinate of any particular monoclonal antibody and/or antibody variant or derivative along a first dimension of said array; Yn is the coordinate of any particular monoclonal antibody and/or antibody variant or derivative along a second dimension of said array; and wherein n is any positive finite number.

It is essential to the present invention to have two high-density arrays, to provide for the detection in parallel of expression levels of proteins present in complex cellular extracts. The predetermined specificity and cross-reactivity of the components of the

primary and secondary arrays provides a unique signal or tag on a single or group of related antigens in the primary array with one or more monoclonal antibodies and/or antibody variants and/or derivatives in the secondary array. This is achieved by high-throughput western blotting of hybridoma supernatants and/or individual lots of one or more phage-derived antibody elements against whole primary arrays, at a rate of about 50,000 to 100,000 per annum.

In general, the primary array is a soup of elements that contains a significant portion of the antigenic diversity encoded by a genome. It is highly reproducible and may be used in the large-scale western blotting of patient sera, in the analysis of protein:protein interactions, in the synthesis of large quantities of recombinant proteins of interest for further study, the development of diagnostic kits and reagents and in the screening of cellular immune responses with respect to individual elements of the array. The soup of elements in the primary array may also be derived from an induced expression library and used as an inoculum for immunisation/vaccination. By virtue of the size and redundancy of the primary array, the elements of the "soup" are present in approximately equimolar concentration and low abundance molecules are up-regulated with respect to their normal abundance in biological samples. Accordingly, the primary array may be used, for example, as a subunit vaccine with Th1 and Th2 and Th1/Th2 inducing elements, based upon the statistical likelihood of occurrence of a particular array element within a whole gene expression library.

In general, the secondary high density protein array , and positive responders to cellular extracts, represent an inexhaustible supply of antibodies of interest which may be stored frozen and provide for the affinity-enrichment from cellular extracts of native proteins of interest for further characterisation, molecular diagnosis and the production of therapeutic magic bullets. The secondary array described herein is not able to be constructed in a high-throughput and effective manner without access to high-density primary arrays.

Genes and/or gene products that correspond to positive responders in either the primary or secondary arrays are included in drug screening programs and/or used in the design of intervention strategies, as will be apparent form the description provided herein.

As used herein, the word "array" shall be taken to mean any ordered arrangement of a plurality of specified integers, including both linear and non-linear arrangements of a plurality of proteins and/or monoclonal antibodies or antibody variants or derivatives. In the present context, the word "array" includes any elements derived from a complex mixture of proteins resolved by 1-dimensional or 2-dimensional gel electrophoresis or chromatography, or peptide or protein expression libraries and the ordered arrangement of proteins or antibodies, antibody variants or derivatives on a grid, such as in microtitre wells or on a membrane support or silicon chip or on a grid comprising a plurality of polymeric pins.

The primary and secondary arrays described herein comprise a plurality of proteins and antibodies, respectively, ordered in two dimensions, wherein the number of proteins or antibodies in each dimension is at least one. Accordingly, the primary and/or secondary array may comprise a single row of a plurality of proteins or antibodies. By "plurality" is meant any large number. Single proteins or antibodies do not constitute an "array" within the context of the present invention, because these do not qualify as a plurality of proteins or antibodies. Nor do single proteins comprise a multiplicity of protein elements designed to emulate a significant portion of the antigenic diversity of a cell, tissue, organ or whole organism.

Each protein within the primary array is assigned a coordinate (Xn,Yn) to facilitate its identification therein. Similarly, each monoclonal antibody or antibody variant or derivative in the secondary array is assigned a coordinate (Xn,Yn) to facilitate its identification therein. The nature of specific antigenic relationships between proteins and antibodies may be determined by analysing the binding of monoclonal antibody or antibody variant or derivative having specific coordinates in the secondary array to

proteins having specific coordinates in the primary array and thereby allow the determination of the abundance of given antigens based on prior knowledge of the antigen-binding capacity of one or more antibodies prior to their exposure to native and/or denatured proteins from cellular extracts.

Figure 1 demonstrates the functionality of parallel screening of a plurality of antigens and mixed abundances and binding affinities to a multiplicity of monoclonal antibodies. As will be apparent to those skilled in the art, such parallel screening represents a significant advance over the prior art, which relies exclusively upon 2-dimensional gel electrophoresis to analyse proteome expression.

The precise number of proteins or antibodies comprising the primary array and secondary array will depend upon the antigenic diversity of the source cell, tissue, organ or organism to which the proteome relates. Thus, for the presently-described array (i.e. both both the primary and secondary arrays), the value of n will vary depending upon the complexity of the proteome of the species from which the array of the present invention is derived. For example, in the case of a bacterium, there may be approximately 4,000 to 20,000 gene products, compared to 100,000 to 300,000 gene products for the human proteome. As will be apparent from the description provided herein, the precise value of n is not essential to the present invention, because technologies may be utilised which provide for a significant portion of the antigenic diversity of any proteome to be represented in array primary array and, as a consequence, for the appropriate number of monoclonal antibodies and/or antibody variants or derivatives to be contained in the secondary array of the present invention.

However, the larger the number of proteins and antibodies represented in both arrays of the present invention, the greater will be its utility in determining the protein profile of a cell, tissue, organ or whole organism.

Preferably, the primary array comprises redundant proteins to increase the likelihood

of emulating the antigenic diversity of the proteome or a significant portion thereof, particularly through the use of one or more protein expression libraries.

Preferably, the secondary array comprises redundant monoclonal antibodies and/or antibody variants and/or antibody derivatives. In secondary arrays, such redundancy increases the number of sites tagged per molecule, to provide increased experimental confidence in the response obtained and, in the case of comparative assays, such as between a biological sample and a biological standard or between a diseased and healthy individual or group of individuals, the potential for a differential response to be detected. Accordingly, redundancy in the secondary array increases the potential to uniquely identify a given antigen by a combination of one or more antibodies, to produce a unique signature for every sample or set of samples assayed.

By "protein profile" is meant the range of expressed proteins of a cell, tissue, organ or organism or any derivative fraction thereof, as distinct from the "proteome" of the cell, which includes the potential protein output of a cell, tissue, organ or organism.

Accordingly, the present invention is particularly useful for the determination of specific proteins that are expressed in cell, tissues or organs of an organism, or whole organisms *per se*, at any time or developmental stage or disease state, by virtue of the provision of both an array that is representative of the proteome of the cell, tissue, organ or whole organism and a method of screening said array.

By "representative of the proteome" means that the array described herein approximates or describes the proteome.

Preferably, the primary array is a redundant array that is representative of a significant portion of the antigenic diversity of a cell, tissue, organ or organism, as exemplified by Figure 2.

By "representative of a significant portion of the proteome" means that the array described herein approximates or describes a significant part or portion of the proteome.

As used herein, the word "protein" shall be taken to mean any molecule that comprises a sequence of naturally-occurring and/or glycosylated and/or acylated and/or non-naturally-occurring amino acid residues, including a fusion protein or a fusion molecule that comprises non-amino acid substituents, such as carbohydrates and/or lipids. Also included within the scope of the definition of a "protein" are recombinant polypeptides, chemically-synthesized peptides (such as produced by Fmoc chemistry), and peptides, oligopeptides and polypeptides derived from a full-length protein by chemical or enzymatic cleavage, using reagents such as CNBr, trypsin, or chymotrypsin, amongst others.

The terms "recombinant polypeptide" and "recombinant peptide" as used herein shall be taken to refer to any polypeptide or peptide molecules that is produced in a virus particle or a cell by the expression therein of a genetic sequence encoding said polypeptide under the control of a suitable promoter, wherein a genetic manipulation has been performed in order to achieve said expression. Genetic manipulations which may be used in this context will be known to those skilled in the art and include, but are not limited to nucleic acid isolation, restriction endonuclease digestion, exonuclease digestion, end-filling using Klenow fragment of *E. coli* DNA polymerase I or T4 DNA polymerase enzymes, blunt-ending of DNA molecules using T4 DNA polymerase or *Exo*III enzymes, site-directed mutagenesis, ligation, and amplification reactions. As will be known to those skilled in the art, additional techniques such as nucleic acid hybridisations and nucleotide sequence analysis, may also be utilised in the preparation of recombinant polypeptide and peptide molecules, in confirming the identity of a nucleic acid molecule encoding said molecules and a genetic construct comprising the nucleic acid molecule.

In one exemplification of the present invention, the proteins of the primary array are

modified by the addition of one or more amino acids to their amino or carboxyl termini. The added amino acids are particularly useful for coupling the protein to another peptide or polypeptide, to a large carrier protein or to a solid support. Amino acids that are useful for these purposes include but are not limited to tyrosine, lysine, glutamic acid, aspartic acid, cysteine and derivatives thereof. Additional protein modification techniques can be used, e.g., NH_2-acetylation or COOH-terminal amidation, to provide additional means for coupling the proteins to another polypeptide, protein, or peptide molecule, or a support. Procedures for coupling proteins to each other, or to carrier proteins or solid supports, are well known in the art. Proteins containing the above-mentioned extra amino acid residues at either the carboxyl- or amino-termini and either uncoupled or coupled to a carrier or solid support, are consequently within the scope of proteins used to produce the primary arrays of the present invention.

In a further embodiment of the present invention, the proteins of the primary array are modified by the addition of one or more reporter molecules, which are bound thereto to facilitate detection of said proteins.

As used herein, the term "reporter molecule" shall be taken to refer to any molecule which is capable of producing an identifiable or detectable result.

Preferred reporter molecules include, but are not limited to, radiochemicals, fluorescent compounds such as rhodamine, biotin, DIG, immunologically-interactive peptides such as FLAG peptides, poly-His amino acid sequence or poly-Lys amino acid sequence or other known amino acid string and functional enzymes, such as alkaline phosphatase, horseradish peroxidase, the *Escherichia coli* β-galactosidase enzyme, the firefly luciferase protein (Ow *et al*, 1986; Thompson *et al*, 1991) and the green fluorescent protein (Prasher *et al*, 1992; Chalfie *et al*, 1994; Inouye and Tsuji, 1994; Cormack *et al*, 1996; Haas *et al*, 1996; *see* also GenBank Accession No. U55762).

Preferably, the primary array will be a high-density protein array. As used herein, the term "high density protein array" means an array comprising at least about 10 proteins per cm^2, more preferably at least about 50 proteins per cm^2, even more preferably at least about 100 protein per cm^2, and still even more preferably at least about 500 proteins per cm^2. Using current robotic technology as applied to, for example the transfer of an induced suspension derived from individual bacterial colonies containing cDNA clones induced, such as by IPTG, to synthesize increased amounts of the protein or peptide encoded therefor, alone or in conjunction with molecular tags, carriers or immunogenic leader sequences, approximately 30,000 protein elements can be transferred at a pitch of approximately 450 microns to solid supports that are positioned within or attached to the lid of a standard 96-well microtitre plate, at a rate of 90 per day, achieved by two gridding robots (KB Engineering, UK; Figure 3). Higher densities can be achieved but the daily production rate is reduced.

Thus, hundreds, preferably thousands, more preferably tens-of-thousands and even more preferably hundreds-of-thousands of proteins may be contained in the primary array. Those skilled in the art will recognise the advantages in terms of time-saving and cost-effectiveness, of providing the primary array in as high a density as is technically-feasible.

By "antigenic diversity" is meant different epitopes (i.e. immunogenic regions) of proteins or peptide fragments or regions thereof.

The term "significant portion of the antigenic diversity" or similar term shall be taken to mean a significant fraction or part of the different linear and conformational epitopes associated with all proteins contained within a cell, tissue, organ or whole organism that is representative of its proteome.

Accordingly, the primary array of proteins comprises a plurality of B cell epitopes. However, since the production of antibodies, including neutralizing antibodies, by B

cells is critically dependent on cognate T cell help, and antigenic determinants recognized by T cells are often distinct from the ones recognized by B cells, identification of T cell epitopes is also important when considering the composition of a significant portion of the antigenic diversity of a cell, tissue, organ or whole organism. Accordingly, it is preferred that the primary array comprises a plurality of B cell epitopes and T cell epitopes.

Primary arrays comprising both B cell epitopes and T cell epitopes permit the identification of protein biological samples that are involved in both cellular and humeral immune responses, for example by using T cell mitogen assays and γ-IFN assays (eg stimulation of nitric oxide production).

Those skilled in the art will be aware that B cell epitopes and T cell epitopes are determined by several intrinsic factors, including for example, accessibility, hydrophilicity and mobility of proteins and/or protein regions/domains.

B cell epitopes are regions of proteins that are cross-reactive with immunoglobulin molecules on B cells. Regions of proteins need to be accessible to immunoglobulin molecules on B cells in order to be immunogenic. Preferably, B cell epitopes will be located on the surface of proteins. Protein folding tends to bury hydrophobic residues in the interior of the folded protein while leaving hydrophilic residues exposed to the aqueous environment. Therefore, hydrophilic regions of proteins are more likely to be immunogenic than hydrophobic ones. Segmental mobility of the polypeptide backbone in regions of proteins is associated with immunogenicity (Tainer *et al.*, 1984), because more mobile regions of proteins are more likely to interact with Immunoglobulin molecules on B cells than less mobile regions.

Various strategies have been developed to locate B cell epitopes in proteins. These strategies include both predictive and non-predictive techniques. Predictive techniques include searches for either hydrophilic or mobile regions of proteins, for example by

examining the primary sequences of proteins, or alternatively, identifying intron-exon boundaries in genes (Tainer *et al.,* 1984). In order to confirm whether a region identified by these techniques is indeed immunogenic, a synthetic peptide is synthesised which contains the predicted amino acid sequence. This synthetic peptide, often coupled to a carrier protein in order to provide T helper cell epitopes, is then used to immunise an animal. Antibodies in the antiserum produced by the animal are then tested for the ability to bind to either the peptide, or the protein, against which it was raised. Antibody binding confirms that the predicted immunogenic region is indeed a B cell epitope.

In the production of the primary array described herein, it is particularly preferred that a non-predictive approach be taken, however a predictive approach can be employed in conjunction with toxic genes and genes corresponding to unstable clones in gene-libraries, for increased coverage or closure in fully-sequenced genomes and/or to increase the redundancy in primary arrays and/or to maximise the number of B cell epitopes represented in the primary array. In this approach, animals are immunised with one or more proteins or whole cell, tissue or organ extracts, in order to generate polyclonal antiserum. This antiserum is then tested for binding to randomly-generated synthetic or recombinant peptides. Various techniques well-known to those skilled in the art have been developed to generate peptide libraries containing peptides of various lengths, such as pentapeptides or hexapeptides (Geysen *et al.,* 1987; Scott and Smith, 1990; Houghton *et al.,* 1991; Lam *et al.,* 1991; du Plessis *et al.,* 1994). These peptides can either contain overlapping sequences from the protein or random sequences. Synthetic or recombinant peptides that bind to the polyclonal serum represent B cell epitopes that are useful in the primary array of the present invention.

T cell epitopes also comprise specific regions of proteins, determined primarily by the specificity of the major histocompatibility complex (MHC) molecules (Schaeffer *et al.,* 1989). Most amino acid sequences recognized by T cells are composed of continuous stretches of peptides (Streitcher *et al.,* (1982); DeLisi and Berzofsky, 1985; Margalit

et al., 1987). Each different MHC molecule binds to a peptide with a different motif, of approximately 8 to 9 amino acid residues, in the case of MHC class I molecules, or 13 to 14 amino acid residues, in the case of MHC class II molecules (Falk *et al.,* 1991; Rudensky *et al.,* 1991). Thus, the ability of an animal's T cells to respond to particular peptides, oligopeptides, polypeptides or proteins, may be assayed to identify T cell epitopes for use in constructing the primary array of the present invention.

To produce the primary array, several sources of protein (i.e. a multiplicity of protein elements) that are representative, collectively or separately, of a significant portion of the antigenic diversity of the cell, tissue, organ or organism are contemplated herein, including, but not limited to one or more peptides, oligopeptide, polypeptide or proteins selected from the list comprising synthetic peptides such as synthetic random amino acid sequences and/or synthetic peptides comprising signature amino acid sequences, and/or recombinant molecules such as those produced in peptide libraries and/or induced peptide expression libraries, such as by protein expression from within one or more cloned gene libraries, and/or naturally-occurring proteins, such as proteins derived from two-dimensional gel electrophoretograms of biological samples and/or naturally-occurring proteins produced in rapidly dividing cells such as by replication-induced protein synthesis (RIPS), amongst others.

Preferably, proteins used to construct the primary array are produced so as to be compatible with the MHC class I and MHC class II molecules, to improve their immunogenicity.

"Synthetic peptides" are non-naturally-occurring proteins as hereinbefore defined that are produced by any method known to those skilled in the art, such as by using Fmoc chemistry.

Synthetic peptides or recombinant peptides generated from expression of cloned nucleic acid inserts are particularly preferred, because they are more time-conserving

and cost-effective to prepare.

Preferred synthetic peptides for use in constructing the primary array will comprise at least 5 amino acid residues in length, more preferably about 5 amino acids to about 80 amino acids in length, and more preferably about 10 amino acid residues to about 20 amino acid residues in length. The final length of peptide used will depend upon the cost of synthesis and immunogenicity of the shorter peptides. However, where the use of shorter peptides is highly desirable (eg. because of budgetary constraints), notwithstanding the reduced immunogenicity of shorter synthetic peptide molecules, then these may incorporate one or more N-terminal or C-terminal basic amino acid residues, in particular one or more lysine residues, to facilitate the formation of a peptide cluster of increased immunogenicity relative to the base peptide, or alternatively, to facilitate conjugation of the peptide to a hapten or other molecule.

Preferably, the synthetic peptides will comprise random amino acid sequences, to maximise the number of sequences represented. Those skilled in the art will be aware that it is possible to chemically synthesize quantities of peptides having random amino acid sequences that can be screened for antigenic determinants. Accordingly, whilst such sequences may not be derived from the proteome of a cell, tissue, organ or organism, they may be useful for "describing" the proteome of the cell, tissue, organ or organism, by virtue of their immunological cross-reactivity to elements of the proteome. Random synthetic peptides are particularly useful as analogues of non-linear (i.e. conformational) epitopes of proteins in the proteome, because such epitopes are generally formed from non-contiguous regions in a protein and the random synthetic peptides provide immunogenic equivalents thereof in the form of a contiguous amino acid sequence. Random synthetic peptides that cross-react with a B cell and/or T cell epitope of the proteome may be identified by screening libraries of such sequences with polyclonal antibodies that bind to T cell and/or B cell epitopes of the proteome, said antibodies being generated, for example, by immunizing animals such as mice or rabbits with whole protein derived from rapidly-dividing cells and/or

tissues or derivative protein fractions thereof. Alternatively, the antibodies used to identify the random synthetic peptides may be monoclonal or recombinant antibodies, in crude or purified form. Those random synthetic peptides that comprise T cell epitopes may be identified by their ability to stimulate T cell cytotoxic or proliferative responses *in vitro*.

Those skilled in the art will be aware that not all proteins in the proteome are equally abundant or equally immunogenic and, as a consequence, polyclonal sera produced against a cell or tissue extract may comprise a skewed distribution of antibody specificities and not be truly representative of the proteome or a part thereof. The use of rapidly-dividing lymphocytes is particularly preferred, because in such extracts there is a greater likelihood that proteins encoded by every open reading frame in the genome of an organism will be represented, by virtue of replication-induced protein synthesis. In preparing the antibodies for screening to identify useful synthetic peptides to incorporate into the primary array, the immunogenic and/or antigenic activity of proteins in the proteome may be increased by their conjugation to haptens, such as KLH, or to substituents that make them less sensitive to enzymatic degradation, and which are more selective. For example, the proline analogue, 2-aminocyclopentane carboxylic acid (βAC^5c), has been shown to increase the immunogenic activity of a naturally-occurring polypeptide more than 20 times (Mierke *et al*, 1990; Portoghese *et al*, 1990; Goodman *et al*, 1987).

In an alternative embodiment, the synthetic peptides will comprise signature amino acid sequences. By "signature amino acid sequence" is meant an amino acid sequence that is representative of a particular class of protein or enzyme (eg. a phosphatase, kinase, helix-loop-helix protein, zinc-finger(III) protein, etc) or an amino acid sequence that is representative of a particular region or domain of a protein (eg. DNA-binding domain, activation region of a transcription factor, ATP-binding site, etc).

The determination of signature amino acid sequences is largely predictive, utilising

computer-driven programmes and algorithms, including the MOTIFS programme of the Computer Genetics Group, Inc., University Research Park, Madison, Wisconsin, United States of America (Devereaux *et al*, 1984), and by the alignment of amino acid sequences present on databases such as the EMBL and GenBANK databases, to determine conserved regions thereof, amongst other approaches. To facilitate such analyses to determine signature amino acid sequences, particularly in cases where multiple amino acid sequences are being compared, the ClustalW programme of Thompson *et al* (1994) can be used.

Alternatively, or in addition, signature amino acid sequences may be determined empirically, using nuclear magnetic resonance spectroscopy (NMR) and X-ray crystallography to identify specific secondary, tertiary or quaternary structures in proteins that comprise substrate-binding domains, B cell epitopes, T cell epitopes, or other functional regions or domains, and then determining the specific amino acid sequences which form such structures. Those skilled in the art will be aware that NMR measures the amount of amino acids as well as the neighbourhood of protons of different amino acid residues, wherein the alternating effect of two protons along the carbon backbone is characteristic of a structural motif.

For example, B cell epitopes may be identified by NMR and/or X-ray crystallographic analysis of antigen:antibody complexes, wherein X-ray techniques require the complex to be crystallized, whereas NMR allows analysis of the complex in a liquid state.

For convenience, linear domains of proteins identified using such empirical approaches are preferred, because synthetic peptides comprising these are readily produced therefrom.

In the case of non-linear domains of proteins, it is necessary to produce a second generation synthetic peptide that mimics the function of the non-linear domain. For

example a second generation synthetic peptide that mimics a B cell or T cell epitope of a protein may be produced by screening synthetic random peptides for antibody-binding activity or T cell proliferative responses, respectively.

Preferably, peptides comprising signature amino acid sequences do not include hydrophobic regions, regions that are likely to be internal to the protein, or regions comprising proline-rich and/or cysteine-rich amino acid sequences.

Peptides comprising signature amino acid sequences may also be produced as recombinant peptides. Recombinant proteins, such as those derived from the expression of cDNA inserts, are capable of emulating many conformational epitopes, however not all as in the native protein.

Recombinant peptides, including those comprising signature amino acid sequences, will preferably be derived from isolated nucleic acid molecules that comprise nucleotide sequences derived from one or more Expressed Sequence Tags (ESTs), amplification products, including PCR products or isothermic amplification products, cDNA sequences, exon regions of isolated genes or random sheared genomic DNA. Such sources of nucleic acid molecules are well-known in the art.

Recombinant peptides may be produced by standard means known to those skilled in the art, the only requirement being that the nucleotide sequence of the nucleic acid molecule encoding the peptide is presented in an expressible format. As used herein, the term "expressible format" shall be taken to indicate that a protein-encoding region of a nucleic acid molecule placed in operable connection with a promoter or other regulatory sequence capable of regulating expression in a cellular or cell-free system.

Preferably, nucleic acid molecule placed in operable connection with a promoter comprises a nucleotide sequence encoding the proteome protein of interest (i.e. the protein to be included in the primary array), in addition to one or more nucleotide

sequences encoding inteins and/or highly immunogenic peptides.

As used herein, the word "intein" shall be taken to mean an excisable protein element or any number of protein purification/enrichment tags linked to a cleavage site for recovery of all or a part of a fusion protein from an affinity column and encoded in or near a vector cloning site. The inclusion of inteins in the recombinant peptide of the present invention is to facilitate the excision of the recombinant proteome protein of interest or a fragment thereof from any other recombinant amino acid sequences that have been co-expressed therewith. More preferably, the nucleotide sequence encoding the recombinant proteome protein of interest or fragment thereof is flanked by nucleotide sequences encoding inteins. This ensures that the recombinant proteome protein of interest or fragment thereof is obtainable as a protein of predictable length following its expression and subsequent purification.

Preferred nucleotide sequences encoding highly immunogenic proteins include those encoding proteins that facilitate Th1;Th2-type and/or Th1/Th2-type responses obtained by cholera toxin, interleukins, or interferon molecules, amongst others. the purpose of including such sequences is to facilitate the primary and secondary antibody responses obtained by immunising animals with the proteome protein of interest, in applications where specific antibodies against said protein are required, and particularly where the ensemble or a multiplicity of the constituent elements used in the construction of primary arrays are employed to engender an immune response.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner.

In the present context, the term "promoter" is also used to describe a recombinant, synthetic or fusion molecule, or derivative which confers, activates or enhances the expression of a nucleic acid molecule which encodes a protein. Preferred promoters can contain additional copies of one or more specific regulatory elements, to further enhance expression and/or to alter the spatial expression and/or temporal expression of the said nucleic acid molecule.

Placing a nucleic acid molecule under the regulatory control of a promoter sequence means positioning the said molecule such that expression is controlled by the promoter sequence. Promoters are generally, but not necessarily, positioned 5′ (upstream) to the genes that they control. In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene from which the promoter is derived. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

The prerequisite for producing intact polypeptides in bacteria such as *E. coli* is the use of a strong promoter with an effective ribosome binding site. Typical promoters suitable for expression in bacterial cells such as *E. coli* include, but are not limited to, the *lacz* promoter, temperature-sensitive λ_L or λ_R promoters, T7 promoter or the IPTG-inducible *tac* promoter. A number of other vector systems for expressing the nucleic acid molecule of the invention in *E. coli* are well-known in the art and are described for example in Ausubel *et al* (1987) or Sambrook *et al* (1989). Numerous plasmids with suitable promoter sequences for expression in bacteria and efficient ribosome

binding sites have been described, such as for example, pKC30 (λ_L: Shimatake and Rosenberg, 1981), pKK173-3 (*tac*: Amann and Brosius, 1985), pET-3 (T7: Studier and Moffat, 1986), the pFLEX series of expression vectors (Pfizer Inc., CT, USA) or the pQE series of expression vectors (Qiagen, CA), amongst others. Typical promoters suitable for expression in viruses of eukaryotic cells and eukaryotic cells include the SV40 late promoter, SV40 early promoter and cytomegalovirus (CMV) promoter, CMV IE (cytomegalovirus immediate early) promoter amongst others.

Means for introducing the isolated nucleic acid molecule or a genetic construct comprising same into a cell for expression are well-known to those skilled in the art. The technique used for a given organism depends on the known successful techniques. Means for introducing recombinant DNA into animal cells includes microinjection, transfection mediated by DEAE-dextran, transfection mediated by liposomes such as by using lipofectamine (Gibco, MD, USA) and/or cellfectin (Gibco, MD, USA), PEG-mediated DNA uptake, electroporation and microparticle bombardment such as by using DNA-coated tungsten or gold particles (Agracetus Inc., WI, USA) amongst others.

In an alternative embodiment of the present invention, proteins for inclusion in the primary array are produced in the form of a recombinant peptide library. As used herein, the term "peptide library" shall be taken to mean the inducible protein products of any set of diverse nucleotide sequences encoding a set of diverse amino acid sequences, wherein said nucleotide sequences are preferably contained within a suitable plasmid, cosmid, bacteriophage or virus vector molecule which is suitable for maintenance and/or replication in a cellular host. The term "peptide library" includes a random peptide library, in which the extent of diversity between the amino acid sequences or nucleotide sequences is numerous, and a limited peptide library in which there is a lesser degree of diversity between said sequences. The term "peptide library" further encompasses random amino acid sequences derived from a cellular source, wherein the amino acid sequences are encoded by a second nucleotide

sequence which comprises bacterial genome fragments, yeast genome fragments, insect genome fragments or compact vertebrate genome fragments, amongst others obtained for example by shearing or partial digestion of genomic DNA using restriction endonucleases, amongst other approaches. A "peptide library" further includes cells, virus particles and bacteriophage particles comprising the individual amino acid sequences or nucleotide sequences of the diverse set.

Preferably, the peptide library is produced as a display library, wherein the protein for inclusion in the primary array is expressed on the surface of a filamentous phage (i.e. a phage display library) which has been introduced into a suitable bacterial host cell, or on polysomes or other suitable display system. Phage display has rapidly matured as a widespread technology for harnessing the chemical and structural diversity of peptide libraries. Using existing phage display technology, it is possible to express one or more recombinant proteins from peptide libraries on the surface of a filamentous bacteriophage, such as M13, as biologically-active or immunologically interactive molecules (for reviews, *see* Burritt *et al.*, 1996; Burton, 1995; Clackson and Wells, 1994; Cortese *et al.*, 1995; Daniels *et al.*, 1995; Lowman, 1997; O'Neil and Hoess, 1995; and Sternberg and Hoess, 1995). Display of peptides and proteins on the surface of bacteriophage. Alternative systems for the display of proteins from combinatorial libraries have also been described (Mattheakis *et al.*,1994; Winter, 1994).

Preferred peptide libraries according to this embodiment of the invention are induced peptide expression libraries.

Alternatively or in addition, preferred peptide libraries according to this embodiment of the invention are "representative libraries", comprising a set of amino acid sequences or nucleotide sequences encoding same, which includes all possible combinations of nucleotide sequences encoding a specified length of peptide.

Preferably, the peptide library comprises cells, virus particles or bacteriophage particles comprising a diverse set of nucleotide sequences which encode the diverse set of amino acid sequences (i.e. the proteome proteins), placed operably under the control of a promoter sequence which is capable of directing the expression of said nucleotide sequence in said cell, virus particle or bacteriophage particle. According to this embodiment of the invention, the nucleotide sequences which encode the proteome proteins are derived from randomly-synthesized oligonucleotides, and more preferably, from randomly-sheared genomic DNA.

It will be apparent from the disclosure herein that peptide libraries, such as those wherein protein expression is from one or more cloned gene libraries, including phage display libraries, may be generated by "shotgun" cloning of pools of said nucleotide sequences into a suitable plasmid vector or other expression vector, such as the λZAP series of vectors (Stratagene, Inc., CA., USA), thereby facilitating the screening of large numbers of peptide-or polypeptide encoding clones in yeast and/or bacterial cells.

Preferably, the nucleotide sequence encoding the proteome protein in an expressible format further includes a sequence targeting the protein to the surface of a cell in which it is expressed (eg. a trans-membrane domain), or alternatively, in the case of virus-mediated or bacteriophage-mediated expression, the proteome protein may be produced as a fusion protein with the coat protein of the virus or bacteriophage, to facilitate immunoreactivity of the expressed proteins using intact cells, virus particles or bacteriophage.

In yet another embodiment of the present invention, the primary array is constructed using naturally-occurring proteins. By "naturally-occurring protein" is meant a protein derived from a virus particle, bacteriophage, cell, tissue, organ or organism, including a partially-purified or purified product.

In one exemplification of this embodiment, array elements derived from protein spots are electro-eluted from two-dimensional gel electrophoretograms of naturally-occurring protein mixtures. In an alternative embodiment, two-dimensional gel electrophoretograms of naturally-occurring protein mixtures are transferred to nitrocellulose or PVDF membranes and individual proteins are excised from the membranes as spots. More preferably, the proteins are transferred with the assistance of robotics (Figure 3), onto grids as high-density arrays.

Proteins initially resolved by the use of "proteomic contigs" (Humphery-Smith and Blackstock, 1997; Humphery-Smith *et al.*, 1997) may also be used in the construction of the primary array, each of which is preferably transferred, more preferably with the assistance of robotics (Figure 3), onto grids, as high-density arrays. A "proteomic contig" is a window of protein expression. Proteomic contigs produced as described herein are collated to represent the entire proteome of an organism or a significant fraction thereof.

The proteins of the primary array are preferably bound to a solid support or matrix to facilitate screening with monoclonal antibodies and/or antibody variants and/or derivatives. The solid support is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs, silicon chips, microplates, polyvinylidene difluoride (PVDF) membrane, nitrocellulose membrane, nylon membrane, other porous membrane, non-porous membrane (eg. plastic, polymer, perspex, silicon, amongst others), a plurality of polymeric pins, or a plurality of microtitre wells, or any other surface suitable for immobilising proteins and/or conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing the protein molecule to the solid support.

Although it is desirable for the antigens to be incorporated on or in the array in similar

quantities, in all cases, it is expected that similar levels of each antigen may be difficult to incorporate on or in gridded arrays, but a response alone or comparatively with respect to test or control groups, for example, may be of considerable importance in detecting immune responses to entire organisms or diseased tissues in association with disease (e.g. cancers) or infection. Reporter molecules bound to the proteins may facilitate normalisation to account for differences in amounts of proteins incorporated on or in the primary array.

The secondary array comprises monoclonal antibodies and/or antibody variants and/or antibody derivatives, optionally bound to a solid porous or non-porous support or matrix and optionally labelled with one or more reporter molecules to facilitate their quantitation and/or detection.

Preferably, the secondary array is an array of monoclonal antibodies and/or hybridomas producing same and/or antibody variants and/or antibody derivatives, that bind to multiple antigenic determinants in the primary array. More preferably, the monoclonal antibodies and/or hybridomas producing same and/or antibody variants and/or antibody derivatives each bind to one or more antigenic determinants on the primary array. The pattern of antigen recognition of such molecules may be determined from data on the coordinates of the proteins in the primary array and of the coordinates of the monoclonal antibodies in the secondary array, and used to elaborate on the nature of shared epitopes in the proteins of the primary array.

Preferably, the secondary array will be a high-density antibody array. As used herein, the term "high density antibody array" means an array comprising at least about 10 monoclonal antibodies and/or antibody variants and/or antibody derivatives per cm^2, more preferably at least about 50 monoclonal antibodies and/or antibody variants and/or antibody derivatives per cm^2, even more preferably at least about 100 monoclonal antibodies and/or antibody variants and/or antibody derivatives per cm^2, and still even more preferably at least about 500 monoclonal antibodies and/or

antibody variants and/or antibody derivatives per cm^2. In a still more preferred embodiment of the invention, the secondary array comprises about 100,000 monoclonal antibodies and/or antibody variants and/or antibody derivatives per cm^2. Thus, hundreds, preferably thousands, more preferably tens-of-thousands and even more preferably hundreds-of-thousands of antibodies may be contained in the secondary array. Those skilled in the art will recognise the advantages in terms of time-saving and cost-effectiveness, of providing the secondary array in as high a density as is technically-feasible.

As used herein, the term "monoclonal antibody" shall be taken to include both an immunoglobulin molecule produced by a hybridoma, and a hybridoma producing one or more immunoglobulin molecules, irrespective of whether or not the specificity of said immunoglobulin molecules is the same. Monoclonal antibodies are obtainable by immunisation with an appropriate gene product, epitope, peptide, or fragment of a gene product, or alternatively, a mixture comprising a plurality of same. Monoclonal antibodies may be selected from naturally occurring polyclonal antibodies raised against one or more epitopes, peptides, or protein fragments, derived from recombinant or naturally-occurring sources.

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an animal immunised with the protein and fused with myeloma cells by standard somatic cell fusion procedures, thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art (*see*, for example Douillard and Hoffman, 1981). For example, the hybridoma technique originally developed by Kohler and Milstein (1975) as well as other techniques such as the human B-cell hybridoma technique (Kozbor *et al.*, 1983), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al.*, 1985), and screening of combinatorial antibody libraries (Huse *et al.*, 1989). Hybridoma cells can be screened immunochemically for production of antibodies which are specifically reactive with the peptide and monoclonal antibodies isolated therefrom.

The term "antibody variant" shall be taken to refer to any synthetic antibodies, recombinant antibodies or antibody hybrids, such as but not limited to, a single-chain antibody molecule produced by phage-display of immunoglobulin light and/or heavy chain variable and/or constant regions, or other immunointeractive molecule capable of binding to an antigen in an immunoassay format that is known to those skilled in the art.

Recombinant antibodies, comprising immunoglobulin light and heavy chain variable and constant regions, expressed on the surface of a bacteriophage or virus particle, such as in a phage display library, are preferred. Optimization of high-affinity antibodies by phage display of combinatorial antibody libraries (Crosby and Schorr, 1995; Winter *et al.*,1994) is a robust mimic of immune selection for natural antibody diversity, as an alternative to traditional hybridoma and immunisation technologies. Human Fabs, single-chain antibodies (de Kruif *et al.,* 1995; Deng *et al.*, 1995; Zdanov *et al.*, 1994), or disulfide-stabilized Fv's (Brinkmann *et al.*, 1995) can be isolated with specificities against virtually any targeted antigen, either foreign or self (Ditzel and Burton, 1995), hapten (Short *et al.*, 1995), carbohydrate (Deng *et al.*, 1995; Zdanov *et al.*, 1994), protein, DNA (Barbas *et al.*, 1995), or RNA (Powers *et al.,*1995). Moreover, cell sub-population-specific monoclonal antibodies may also be derived from synthetic phage antibody libraries (de Kruif *et al.*, 1995). Techniques for the production of recombinant antibodies in phage display libraries are well-known in the art (Crosby and Schorr, 1995; Winter *et al.*,1994).

Several bacteriophage-based vector systems are available for expressing immunoglobulins in phage display libraries, for example the λImmunoZAP vector series including λImmunoZAP L , λImmunoZAP H, λImmunoZAP H/L and λSurfZAP, for expressing immunoglobulin Fab fragments, light chains and heavy chains on the surface of a filamentous phage (Hogrefe *et al.*,1993; Mullinax *et al.*,1990; Shopes, 1992; Stratagene, CA., USA).

In use, the primary arrays of protein, both redundant and non-redundant, can be constructed as described *supra*, and used to dissect in parallel the monoclonal elements from within a polyclonal response in animal-based or phage-based systems, concomitantly with determining the specificity of the antibodies produced from said animal-based or phage-based systems. For example, biopanning of phage-based antibody libraries may be performed, essentially as shown in Figure 1.

The term "antibody derivative" refers to any modified antibody molecule that is capable of binding to an antigen in an immunoassay format that is known to those skilled in the art, such as a fragment of an antibody (Fab fragment), or an antibody molecule that is modified by the addition of one or more amino acids or other molecules to facilitate coupling the antibodies to another peptide or polypeptide, to a large carrier protein or to a solid support (eg. the amino acids tyrosine, lysine, glutamic acid, aspartic acid, cysteine and derivatives thereof, NH_2-acetyl groups or COOH-terminal amido groups, amongst others).

Preferably, the antibody molecules or antibody variants or derivatives of the secondary array are modified by the addition of one or more reporter molecules, which are bound thereto to facilitate their detection.

According to this embodiment, it is possible to detect one or more antigenic determinants in a labelled mixture of antigens, by a variety of means including fluorescence, chemiluminescence, isotopic determination, enzymatic labelling, amongst others.

Preferred reporter molecules for binding covalently or non-covalently to antibodies include, but are not limited to, radiochemicals, fluorescent compounds such as rhodamine, biotin, DIG, immunologically-interactive peptides such as FLAG peptides, poly-His or poly-Lys amino acid sequences or other known amino acid string, protein

A, lectins (eg. phytohemagglutinin A), secondary antibodies and functional enzymes, such as alkaline phosphatase, horseradish peroxidase, the *Escherichia coli* β-galactosidase enzyme, the firefly luciferase protein (Ow *et al*, 1986; Thompson *et al*, 1991) and the green fluorescent protein (Prasher *et al*, 1992; Chalfie *et al*, 1994; Inouye and Tsuji, 1994; Cormack *et al*, 1996; Haas *et al*, 1996; *see* also GenBank Accession No. U55762).

In the case of reporter molecules comprising immunologically-interactive peptides or functional enzymes, these are preferably linked to recombinant antibodies by producing fusion proteins comprising both the immunoglobulin and reporter molecule moieties, wherein the corresponding gene regions encoding said moieties are spliced together in-frame and the recombinant nucleic acid molecule is expressed in a suitable cellular, viral or bacteriophage expression system. Techniques for producing such fusions molecules are well-known in the art.

The monoclonal antibodies and/or antibody variants and/or antibody derivatives used in the construction of the secondary array may be derived from monoclonal or polyclonal antibody sources. For example, nucleotide sequences encoding immunoglobulin variable light and heavy chains may be derived from hybridomas or lymphocytes derived from animals that have been previously immunised with antigen, as for the production of monoclonal or polyclonal antibodies. Conventional immunisation strategies may be utilised to facilitate polyclonal or monoclonal antibody production, or the production of antibody derivatives, including the immunisation of animals, in particular rabbits or mice, with:

(i) whole cells, tissues, organs or whole organisms or sub-cellular fractions or lysates, extracts or other derivatives thereof; and/or

(ii) DNA derived from biological samples corresponding to the whole or fractions of cells, tissues, or organisms and lysates thereof; (i.e. DNA vaccination); and/or

(iii) proteins derived from a biological sample that has been subjected to a

separative procedure, such as 2-dimensional gel electrophoresis; and/or

(iv) synthetic or recombinant peptides comprising signature amino acid sequences; and/or

(v) synthetic or recombinant peptides encoded by open reading frames of an EST, exon sequence, cDNA molecule or other open reading frame of an organism; and/or

(vi) a "soup" comprising the elements used to produce the primary array or a multiplicity or a part thereof.

Best estimates for the primary antibody response in mice is for a total potential repertoire including junctional diversity, but not somatic mutations during a secondary response, of about 109 to about 1011. Phage-based antibodies are likely to be capable of specifically detecting even greater numbers of antigens, however, this capacity for diversity brings with it the need to screen large numbers of clones. Accordingly, immunisation of animals, followed by more traditional monoclonal antibody production and screening, is initially the preferred means for constructing the secondary arrays, but both are employed with a view to increasing the redundancy and multiple signals per target antigen. This allows for signal verification; a combination of responses producing a unique signal or tag on a single or group of related antigens; multiple tag sites along a target antigen to display the differences between healthy and diseased and/or between one or more biological samples and a biological standard.

The present inventors contemplate that, for a small bacterial proteome comprising about 4,000 antigens, up to 12-20 mice will be required to produce sufficient monoclonal antibodies against complex protein mixtures to represent at least one epitope per protein, for 25-75% of the total proteome. The remaining monoclonal antibodies or antibody variants or derivatives will be derived from antibodies against single proteins. Alternatively or in addition, additional mice may be immunised. Naturally, in the case of more complex proteomes, such as the human proteome, more than 12-20 mice, and in particular, up to 100 mice, more preferably up to about 500

mice, may be used to produce sufficient monoclonal antibodies against complex protein mixtures to represent 25-75% of the total proteome. In addition, primary arrays are exposed to ever-increasing numbers of antibody derivatives by high-throughput western blotting, so as to increase redundancy within the primary array and thereby better emulate the antigenic diversity seen in the proteome of the cell, tissue, organ or whole organism.

To obtain the monoclonal or polyclonal antibodies for use in constructing the secondary array, a good immunogenic response is required to ensure that said antibodies have the ability to bind specifically (i.e. uniquely) or non-specifically (i.e. not necessarily uniquely), to individual epitopes in proteins of the primary array. In this regard, the specific or non-specific binding of the antibodies to one or more proteins of the primary array provides combined information or binding attributes in the determination of the protein profile of a cell, tissue, organ, or organism.

The progress of immunization can be monitored by detection of antibody titres in plasma or serum. Standard ELISA or other immunoassay can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, IgG molecules corresponding to the polyclonal antibodies can be isolated from the antisera.

As with all immunogenic compositions for eliciting antibodies, the immunogenically effective amounts of the immunising protein must be determined empirically. Factors to be considered include the immunogenicity of the native peptide, whether or not the peptide will be complexed with or covalently attached to an adjuvant or carrier protein or other carrier, the route of administration for the composition, (i.e. intravenous, intramuscular, subcutaneous, *etc.*), and the number of immunizing doses to be administered. Such factors are known in the vaccine art and it is well within the skill of immunologists to make such determinations without undue experimentation.

In preparing the antibodies, it is particularly preferred to immunize mice with mixtures of antigens, more preferably at least about 10 to about 100 antigens simultaneously.

More preferably, well-expressed antigens associated with improved immunogenicity in such antigen mixtures are expressed at approximately equimolar ratios. Alternatively or in addition, most low-abundance antigens in these mixtures, which correspond to poorly-expressed proteins in naturally-occurring mixtures of cellular antigens, are better-expressed (As will be known to those skilled in the art, because of their low intra-cellular abundance, these antigens are often poorly immunogenic.) To achieve this end, it is desirable to increase maximally the number of hybridomas screened in responder mice (routinely about 600 to about 1,000 hybridomas per mouse), as determined by response levels to the composite antigen mixture (as in the accompanying Examples and as outlined in Figure 4) obtained from each immunised mouse and the antibodies are screened against the primary protein arrays constructed as described *supra*. Any conventional immunoassay format may be used to screen the primary arrays (eg. ELISA, RIA, western blot and the like). Those hybridomas that produce antibodies binding to one or more antigens can be employed to generate a unique and/or desirable recognition for use in screening biological material of interest.

The screening of hybridomas or other antibody-producing cells or virus or phage particles, is completed when monoclonal antibodies or antibody variants or derivatives have been identified that bind to a useful portion of the primary array or uniquely to facilitate identification of any protein derived from a multiplicity of open reading frames, or uniquely identify all protein isoforms encoded or produced by a genome. As the number of elements increases, then so too does the utility of the secondary array. Once completed, the monoclonal antibodies or antibody variants or derivatives are ordered into a secondary array. Thus, the secondary array may be constructed methodically, adding batches of antibodies to pre-existing arrays, to form new, more complete and/or more redundant secondary arrays as each new or monoclonal antibody or antibody variant or derivative is detected.

It is preferred for the monoclonal antibodies or antibody variants or derivatives of the secondary array to be coupled to a solid support. The nature of the solid support used and the mode of binding monoclonal antibodies or antibody variants or derivatives of the secondary array to the solid support is the same as for the proteins of the primary array, as described *supra*.

The antibodies of the secondary arrays are also useful for the affinity-purification or enrichment of proteins to which it binds, either as a single purification step or alternatively, in combination with other known procedures for the purification of proteins. In general, proteins may be purified based upon their size, charge or ability to bind specifically to antibodies against the intact polypeptide, using one or a combination of gel electrophoresis, size-exclusion chromatography, reverse phase chromatography, ion-exchange chromatography or affinity chromatography. After purification/enrichment, a reduced number of protein bands or spots should be detectable with one-dimensional or two-dimensional gel electrophoresis. Methods for the affinity purification of proteins using antibodies are well-known to those skilled in the art. Importantly, the present invention will provide for the purification/enrichment of native proteins from a cellular soup, to facilitate further protein characterisation by any number of analytical and/or structural methodologies well-known to those skilled in the art.

The N-terminal or total sequencing of the isolated protein may also be carried out. This provides the possibility to compare the sequence of the protein with known proteins in databases and may also be arrived at by analysis of cDNA clones encoding elements used in the construction of primary arrays (eg. by analysis of cells expressing induced proteins in solution or on membranes overlaid on agar and lysis of said cells to allow access to the antigens therein).

A second aspect of the present invention clearly encompasses the above-mentioned

secondary array, when produced by the method of:

(i) preparing a primary array of proteins $a^1_{(Xn,Yn)}$, $a^2_{(Xn,Yn)}$, $a^3_{(Xn,Yn)}$,.....,$a^n_{(Xn,Yn)}$ comprising a multiplicity of protein elements designed to emulate a significant portion of the antigenic diversity of a cell, tissue, organ, organism from which the biological sample is derived, wherein $a^1,a^2,a^3,....,a^n$ are proteins; Xn is the coordinate of any particular protein along a first dimension of said array; Yn is the coordinate of any particular protein along a second dimension of said array; and wherein n is any positive finite number;

(ii) screening the primary array with a plurality of monoclonal antibodies and/or antibody variants and/or derivatives one-at-a-time, or a reduced pool thereof one-at-a-time, so as to determine those antibodies and/or antibody variants or derivatives that bind to one or more proteins in said primary array; and

(iii) preparing said secondary array of monoclonal antibodies and/or antibody variants or derivatives using those monoclonal antibodies and/or antibody variants or derivatives that bind specifically or non-specifically to one or more proteins in the primary array.

Conventional immunoassays, such as ELISA, RIA, western blot immunoelectrophoresis or rocket immunoelectrophoresis, amongst others, can be used to perform the screening of the primary array according to this embodiment of the invention.

A wide range of immunoassay techniques are available as can be seen by reference to US Patent Nos. 4,016,043, 4,424,279 and 4,018,653. These, of course, include both single-site and two-site or "sandwich" assays of the non-competitive types, as well as competitive binding assays. The direct binding of a labelled antibody to the target protein in the primary array is also encompassed by the present invention. It will be readily apparent to the skilled technician how to modify or optimise standard immunoassays to perform this embodiment of the present invention and all such

modifications and optimisations are encompassed by the present invention.

Sandwich assays are among the most useful and commonly used assays and are favoured for use in the present invention. A number of variations of the sandwich assay technique exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay as applied to the present invention, an unlabelled protein of the primary array (the primary array *per se*), immobilised on a solid substrate, is brought into contact with the monoclonal antibody or antibody variant or antibody derivative to be tested. After a suitable period of incubation, for a period of time and under conditions sufficient to allow formation of an antibody-antigen complex, a second antibody specific to the first-mentioned antibody, labelled with one or more reporter molecules capable of producing a detectable signal, is then added and incubated, allowing time sufficient for the formation of another complex of antigen-antibody-labelled antibody. Any unreacted material is washed away, and binding of the monoclonal antibody or antibody variant or antibody derivative to the protein of the primary array is determined by observation of a signal produced by the reporter molecule.

Variations on the forward assay include a simultaneous assay, in which both the protein of the monoclonal antibody or antibody variant or antibody derivative and the labelled antibody are added simultaneously to the bound protein.

The most commonly used reporter molecules are enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes), bioluminescent and chemiluminescent molecules.

In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognised, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include

horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable colour change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first protein-antibody complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the tertiary antigen-antibody-antibody complex. The substrate will react with the enzyme linked to the second antibody, producing a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample.

Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic colour visually detectable with a light microscope. As in enzyme immunoassays (EIA), the fluorescent labelled antibody is allowed to bind to the first protein-antibody complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength the fluorescence observed indicates the presence of the hapten of interest. Immunofluorescence and EIA techniques are both very well established in the art and are particularly preferred for the present method.

The results may either be qualitative, by simple observation of the visible signal produced by the reporter molecule, or may be quantitated by comparing with a control sample containing known amounts of protein.

The solid surface to which the protein or monoclonal antibody or antibody variant or antibody derivative is bound is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking, covalent binding or physical adsorption.

Those skilled in the art will be aware that the binding that is detected in any immunoassay, including those applied to the screening of the primary array of the present invention, are susceptible to variation from at least two sources:

(i) differential amounts of protein (antigen) bound to the solid support in the immunoassay; and

(ii) differential association and dissociation constants of antibodies for their specific antigens in the binding reaction.

Accordingly, a further embodiment of the present invention provides for the correction of concentration-dependent variation in signal intensity at the stage of screening the primary array with antibodies to construct the secondary array.

Preferably, this is achieved by labelling the proteins of said array with one or more reporter molecules, for example prior to their attachment to the solid support or matrix, and determining the signal obtained, which is representative of the amount of protein bound to said solid support or matrix. Following screening of the labelled primary array with an antibody against the reporter molecule and quantitation in the manner described *supra*, the signal obtained from the reporter molecule and the signal obtained in the immunoassay are compared. The signal intensity obtained for the immunoassay is then adjusted to account for the concentration of protein, as determined by the signal intensity obtained using the reporter molecule.

Suitable reporter molecules for detecting the amount of protein bound to the solid support include immunogenic peptides or protein regions, such as but not limited to a FLAG peptide, poly-His or poly-Lys amino acid sequence or other known amino acid string. The use of such amino acid sequences is particularly preferred wherein the protein of the primary array is a recombinant or synthetic protein, because the immunogenic peptide or protein region can be produced as an in-frame fusion with said recombinant or synthetic protein. Naturally, the preferred mode of detection of immunogenic peptide reporter molecules is the use of an antibody molecule that specifically binds to such sequences, in which case a standard immunoassay format may be employed.

The secondary array is used to screen complex mixtures of cellular proteins, such as those labelled fluorescently, isotopically, enzymatically, or by other means known to those skilled in the art, to determine the level of binding specificity and/or cross-reactivity to antigens on the solid support, in a standard immunoassay format.

Such immunoassays can be conducted simultaneously with an immunoassay to screen the primary array with the monoclonal antibody or antibody variant or derivative, as the case may be. However, it is preferred that two distinct second antibodies are used, which bind specifically to either the antibody against the reporter molecule or to the antibody being screened, but not to both, and that each second antibody is labelled with a different reporter molecule, to facilitate separation of the signals obtained.

Alternatively, any other suitable reporter molecule described herein may be used to label the proteins of the primary array, subject to the proviso that said reporter molecule is distinct from that used to detect the binding of the monoclonal antibody or antibody variant or antibody derivative to said protein.

The screening of the primary array may also be normalised to reduce or remove variation arising from differential association and dissociation constants of antibodies

in the binding reaction, by averaging the signal intensity obtained, wherein it is known from the screening conducted that several antigenically-distinct monoclonal antibodies or antibody variants or antibody derivatives bind to the same protein. This embodiment highlights the advantages associated with using several monoclonal antibodies and/or antibody variants or derivatives that bind to different epitopes on the same protein.

The array of the present invention is particularly useful for the purpose of determining the protein profile of a biological sample derived from an organism which is the same as, or closely-related to, the organism in respect of which said array is representative of the proteome. This is because an array of the present invention prepared with a specific organism in mind is representative of a significant portion of the antigenic diversity of that organism and, as a consequence, may be used to ascertain any sub-set of that organism's proteome, such as the protein profile of a particular cell, tissue, or organ, either in a healthy or diseased state, or following exposure of a particular cell, tissue, organ or whole organism to an external stimulus, such as a chemical compound or a biological agent (eg. a fungal or viral pathogen). As will be readily apparent from this discussion, the present invention has particular utility in the medical and pharmacological fields, for determining alterations in protein profiles of patients suffering from particular diseases or infections or alternatively, to monitor changes in protein profiles during treatment with drugs.

In performing this embodiment of the invention, the secondary array described *supra* is screened with said biological sample, to determine those proteins therein which bind to one or more monoclonal antibodies and/or antibody variants or derivatives in said secondary array in a uniquely-definable manner.

The biological sample may be any lysate or extract of a cell, tissue, organ, or whole organism, or a derivative fraction thereof, the only requirements being that said biological sample is in a form suitable for conducting an immunoassay (eg. correct pH and buffer composition) and further, that said bioloigcal sample is derived from an

organism that is sufficiently closely-related to the organism against which the array was prepared to be immunologically cross-reactive with the antibodies of the secondary array.

Particularly preferred biological samples according to this embodiment include bodily fluids such as tears, saliva, urine, semen or other exudate, blood, serum, or tissue biopsies comprising skin, heart, liver, kidney, lung, intestine, colon, or foetal cells, or protein extracts derived from of any one or more of said samples. Other biological samples are also encompassed by the present invention, which is generally applicable and not to be limited by the source of biological sample being assayed, or the purpose for which the subject assay is being performed.

Screening of the secondary array may be performed using a standard immunoassay format, similar to that described *supra*. However, the direct binding assay is particularly preferred, wherein the biological sample is brought into contact with the secondary array for a time and under conditions sufficient for an antibody-protein complex to occur and the complex is then washed to remove unbound components.

Preferably, the proteins in the biological sample are labelled with a suitable reporter molecule to facilitate their detection following binding. The reporter molecule may be any reporter molecule that can be routinely conjugated to proteins in complex protein mixtures or alternatively, the proteins may be labelled after the unbound molecules have been washed away. For convenience, fluorescent compounds, such as fluorescein and rhodamine, or radioisotopes are preferred.

Alternatively or in addition, NMR, circular dichroism, changes in electric current, X-ray diffraction or laser technology, may be used to detect binding of the proteins to the antibody molecules.

Positive signals following the binding and washing steps comprise specific proteins

bound specifically to monoclonal antibodies or antibody variants or derivatives in the secondary array. Knowledge of the proteins present in the biological sample is then obtained by reference to the protein in the primary array against which the detected monoclonal antibodies or antibody variants or derivatives in the secondary array was selected, based upon its specific binding thereto. This is achieved by reference to the coordinates (Xn,Yn) of those proteins in the primary array which bind to one or more monoclonal antibodies and/or antibody variants or derivatives in the secondary array.

Alternatively, as mentioned *supra*, interesting proteins that are detected may be purified from biological samples by standard procedures and optionally, the amino acid sequences of such purified proteins and/or post-translational modifications of the isolated proteins may be determined. Additionally, the amino acid sequence of one or more proteins identified in the primary array may be determined if it is not already known.

Additionally, through access to corresponding reactive protein or proteins in the primary array, the nucleotide sequence of DNA encoding said proteins may be determined using standard procedures. The isolated DNA may then be used to produce recombinant proteins that were employed in the primary array, again using standard procedures known to those skilled in the art.

As with the screening of the primary array, the screening of the secondary array with a biological sample can be susceptible to variation from differential amounts of antibody bound to the solid support in the immunoassay or from differential association and dissociation constants of antibodies for their specific antigens in the binding reaction.

Accordingly, a further embodiment of the present invention provides for the correction of concentration-dependent variation in signal intensity at the stage of screening the screening the secondary array with the biological sample.

Preferably, said normalisation is achieved by screening the secondary array with one or more reporter molecules that bind to all monoclonal antibodies and/or antibody variants or derivatives in said array, comparing the signal obtained using said reporter molecule to the signal obtained in the immunoassay using the biological sample, and finally, adjusting the signal intensity obtained using the biological sample to account for the amount of antibody bound as determined by the signal intensity obtained using the reporter molecule.

Molecules that bind generally to antibodies are well-known in the art, and include protein A, lectins and secondary antibodies. Wherein second antibodies are used to detect both the amount of antibody bound to the support and the binding of the proteins in the biological extract to the secondary array antibodies, it is preferred that each second antibody is labelled with a different reporter molecule, to facilitate separation of the signals obtained.

As with normalisation of the primary array to account for different association/dissociation constants of antibodies/antigens, the screening of the secondary array may be normalised by averaging the signal intensity obtained for binding of the same protein to several monoclonal antibodies and/or antibody variants or derivatives that bind to different epitopes on the same protein, which binding would become apparent from “keying” the coordinates of the antibodies bound by the sample back to the primary array.

A variation of the screening strategy described *supra* may be applied to the comparison of biological samples, for example in applications wherein it is important to ascertain the differential expression of specific proteins of the proteome. A significant application of the present invention is the comparison of healthy and diseased tissues from humans and other animals and organisms, or to monitor the effects of any chemicals administered to humans or other organisms, such as during treatment of a specific disease to determine the efficacy of treatment. The present

invention provides a significant contribution to the characterisation of multigenic traits and other multiprotein phenomena that are aetiologically-associated with disease, such as the major diseases of humans or other organisms (eg. one or more of the following: cancer, genetically-inherited disorders, autoimmune disorders, infections and environmental tissue damage heart disease, amongst others).

Alternatively, the inventive method may be applied to the diagnosis or determination of an immune response in a human or animal subject, wherein the biological sample assayed using the array comprises blood or serum or a fraction or derivative of each thereof. Such information may be particularly important in the casse of diagnosing autoimmune diseases or alternatively, past or present infections of host animals by pathogens.

To perform this embodiment of the present invention, the same organism-specific secondary array is screened separately as described herein, with two or more biological samples and the signals obtained using each of said biological samples is compared, to determine those proteins which are differentially expressed. The determined proteins may be further characterised for their association with the specific phenotype being analysed. The phenotype being assayed may be any multiprotein-based phenotype, however the present invention can, of course, be applied to the analysis of single-protein-based phenotypes/phenomena in biological systems.

As stated already, the present invention is particularly useful for analysing multiprotein-based disease states in humans and other organisms. Accordingly, a further aspect of the present invention provides a method of diagnosing a human or animal subject for a medical condition, ailment, illness or immune response or a predisposition for said medical condition, ailment or illness, said method comprising screening the array of the present invention with a biological sample derived from said subject and comparing the protein profile thereof with the protein profile of a biological standard derived from a healthy individual, wherein differences between the biological sample the biological

standard are indicative of said medical condition, ailment, illness or predisposition.

The biological standard may pre-determined or alternatively, screening of the array with the test sample and the standard may be conducted simultaneously. Preferably, the biological standard is derived from the same cell-type, tissue-type, organ-type, bodily fluid-type, blood-type, or serum-type as the biological sample being tested.

The present invention clearly encompasses the steps of obtaining the biological sample prior to screening and/or preparing the array for screening with the biological sample and/or the biological standard. According to this embodiment, the array can be prepared for screening, by selecting a sub-set of monoclonal antibodies or antibody variants that bind to proteins in the primary array derived from a healthy individual and from the same cell-type, tissue-type, organ-type, bodily fluid-type, blood-type, or serum-type as the biological sample. In this manner, any differences in binding that are observed when the sub-set is screened with the biological test sample will become evident immediately.

The diagnostic procedures described herein will elucidate particular proteins associated with disease states in humans and other animals, which proteins may be used to produce vaccine compositions or to identify compounds that correct the altered protein expression of the diseased individual.

Accordingly, a still further aspect of the invention extends to compositions for the therapeutic or prophylactic treatment of a human or other animal subject, said compositions comprising a suite of protein elements and/or responsive antibody elements of relevance to disease genesis and/or disease susceptibility that have been identified by screening the primary and/or secondary array of the present invention and preferably subsequently isolated, in combination with a pharmaceutically-acceptable carrier or diluent.

According to this aspect of the invention, it is particularly preferred that the active ingredient of such compositions is a composite of the multiplicity of elements employed in the construction of the primary array, used in approximately equimolar ratio at a sufficiently-high concentration of each individual protein component to produce an antibody response to each of said protein components.

Preferably, the immune response is a humoral and/or cellular immune response to each of the protein components forming the active ingredient, in a subject to which said composition is administered, rather than merely a response to a few proteins in a naturally-occurring cellular soup.

As used herein "pharmaceutically acceptable carrier and/or diluent" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

Wherein the compositions are used for prophylaxis, it is particularly preferred that they produce an antibody response or protective immune response when administered.

Preferably, the composition elicits or stimulates an immune response when administered to a subject in need of treatment. More preferably, the immune response is a primary or secondary antibody response. Still more preferably, the immune response against the protein or antibody is a protective immune response.

Wherein the subject compositions are intended for therapeutic treatment, they are administered to the subject for a time and under conditions sufficient for the symptoms of said medical condition, ailment, or illness to abate.

Compositions for eliciting immune responses may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intramuscular, subcutaneous, intranasal, intradermal or suppository routes or implanting (eg using slow release molecules). Depending on the route of administration, the immunogenic proteins contained therein may be required to be coated in a material to protect them from the action of enzymes, acids and other natural conditions which otherwise might inactivate said immunogen. In order to administer the compositions by other than parenteral administration, they will be coated by, or administered with, a material to prevent its inactivation. For example, the immunogenic protein may be administered in an adjuvant, co-administered with enzyme inhibitors or in liposomes.

An "adjuvant" as used herein is to be taken in its broadest sense and includes any immune-stimulating compound, including a cytokine molecule , resorcinol, a non-ionic surfactant such as polyoxyethylene oleyl ether or n-hexadecyl polyethylene ether, amongst others.

Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol.

Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes.

The compositions of the present invention may also be administered parenterally or intraperitoneally. Dispersions of the immunogenic protein component can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations can contain a preservative to prevent the growth of microorganisms.

The forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must

be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi.

The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. Fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the immunogenic protein component in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by heat-sterilisation, irradiation or other suitable sterilisation means. Generally, dispersions are prepared by incorporating the various sterilised active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the compositions are suitably protected as described above, they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or enclosed in hard or soft shell gelatin capsule, or compressed into tablets, or

incorporated directly with the food of the diet.

For oral administration, the compositions may be admixed with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of immunogenic protein in such vaccine preparations is such that effective immunisation will be achieved with between one and five doses of said vaccine.

The tablets, troches, pills, capsules and the like may also contain the following: A binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such a sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the compositions may be incorporated into sustained-release preparations and formulations.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the human or animal

subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutically acceptable carrier.

The scope of the invention will be illustrated further by reference to the following non-limiting Example.

EXAMPLE 1
DISSECTING THE POLYCLONAL RESPONSE

Figure 5 is a schematic representation showing the dissection in parallel of the monoclonal elements from within a polyclonal response, for the screening of viable B cell hybridoma culture supernatants. The scheme presented therein, which depends upon a predetermined knowledge of monoclonal antibody specificity, is also applicable to phage-based antibody screening and biopanning as described herein.

This example demonstrates this principle of the present invention, in particular the potential for the invention to generate in parallel a good immune response sufficient to allow the generation of multiple useful specific antibodies and to provide for the screening of at least one order of magnitude of antigens in parallel, thereby facilitating a higher throughput and reduced time and costs for the generation of monoclonal antibodies and costs associated with screening antigens and/or antibodies in parallel, than is provided by the prior art.

The process described herein is applicable irrespective of the number of antigens being employed, including those comprising two, three, four or more orders of magnitude of complexity above that described herein.

Accordingly, the examples described herein, which produce antibodies in a high throughput setting, demonstrate the general applicability of the invention to the screening of the output of the human genome. The reduced time and cost associated with the present invention is greatly facilitated by the combination of steps described herein, that test initially for specificity and subsequently, for cross-reactivity or degree of specificity, the latter often being the greatest cost element in monoclonal antibody production by methods which involve either experimental animals or phage-derived molecular procedures. Additionally, hybridomas or phage-derived antibody derivatives are not currently screened against antigens that are representative of significant

portions of the antigenic diversity associated with a genome and, if so, in the case of tissue sections or expression libraries, the antigens are not contained within retrievable and reproducible data coordinates, in contrast to the dimensional coordinates of the arrays of the present invention exemplified herein. Thus, neither the cross-reactivity nor specificity achievable using the present invention can be achieved by known screening procedures, at the same level of precision as that exemplified herein.

An immunogen comprising 50 μg of a mixture of 12 antigens in equimolar concentration was used to immunise four (4) mice (2 x Balb/C, 2 CBA), in the presence of adjuvant. Approximately one month later, mice were boosted with 25 μg antigen mixture plus adjuvant. Nine days later, mice were bled to obtain serum. The composition of the immunisation mixture used is listed in Table 1.

Bleeds obtained from the immunised mice were screened by ELISA against each of the 12 individual antigens contained in immunisation mixture. Briefly, ELISA plates were coated with individual antigens at levels of 10 μg/ml and 1 μg/ml. The antigens were diluted in a standard carbonate coating buffer at pH 9.6 prior to coating and 50 μl of antigen solution at the appropriate concentration was added to each microtitre well, and incubated overnight at 4°C. The microtitre wells were then blocked for 1 hour at ambient temperature with 0.2%(w/v) casein in phosphate-buffered saline solution (PBS). The plates were washed 3 times in PBS/tween and 50 μl serum from individual mice, titrated 1:50 - 1:400 (2-fold) in TBS/BSA/tween was added to each well and incubated for 1 hour at 37°C. Plates were washed as before and 50 μl of a 1:4,000 dilution of anti-mouse IgG conjugated to horseradish peroxidase (HRP; Silenus product code DAH) in TBS/BSA/tween was added and incubated for a further 1 hr at 37°C. Plates were again washed as before. OPD substrate was added and plates were incubated for 10 minutes at ambient temperature, and the absorbance at 492 nm was determined. Data are presented in Table 2.

The response levels of each mouse to each antigen in the mixture is summarised in Table 3.

TABLE 1

Protein	SIGMA cat code	Molecular Mass	Qty used (μg)
Aprotinin, bovine lung	A1153	6500	6.85
α -Lactalbumin, bovine milk	L5385	14200	15
Lysozyme, chicken egg white	L6876	14300	15.1
Trypsin inhibitor, soybean	T9003	20000	21.1
Trypsinogen, bovine pancreas	T1143	24000	25.3
Carbonic anhydrase, bovine ertyrocytes	C2522	29000	30.6
Glyderaldehyde phosphaste 3 dehyrogenase, Rabbit muscle	G0763	36000	37.9
Ovalbumin, chicken egg	A2512	45000	47.4
Fumarase, procine heart	F1757	48500	51.1
Glutamic dehyrdrogenase, bovine liver	G7882	55000	158
Albumin, bovine serum	A2153	66000	69.5
Galactosidase, E.coli	G2513	116000	122
Total			**499.85**

TABLE 2

Antigen 1 - Aprotinin						
Antigen level	**Serum Dilution**	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5 (Normal mouse serum)**
10 μg/ml	1:50	0.097	0.095	0.174	0.217	0.100
	1:100	0.052	0.065	0.145	0.159	0.054
	1:200	0.033	0.043	0.083	0.093	0.034
	1:400	0.022	0.024	0.056	0.048	0.018
1 μg/ml	1:50	0.123	0.124	0.362	0.189	0.081
	1:100	0.079	0.076	0.295	0.121	0.049
	1:200	0.047	0.042	0.175	0.071	0.037
	1:400	0.023	0.028	0.112	0.037	0.021
Antigen 2 - Lactalbumin						
Antigen level	**Serum Dilution**	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5 (Normal mouse serum)**
10 μg/ml	1:50	0.127	0.124	0.222	0.113	0.086
	1:100	0.090	0.118	0.166	0.110	0.048
	1:200	0.077	0.073	0.096	0.088	0.034
	1:400	0.059	0.068	0.063	0.062	0.019
1 μg/ml	1:50	0.117	0.120	0.457	0.117	0.081
	1:100	0.070	0.056	0.216	0.080	0.059
	1:200	0.056	0.037	0.189	0.041	0.032
	1:400	0.030	0.013	0.080	0.034	0.023

Antigen 3 - Lysozyme						
Antigen level	**Serum Dilution**	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5 (Normal mouse serum)**
10 μg/ml	1:50	1.598	1.573	2.225	1.436	0.094
	1:100	1.395	1.840	2.128	1.465	0.064
	1:200	1.227	1.099	1.920	1.288	0.042
	1:400	0.848	.0779	1.588	0.910	0.024
1 μg/ml	1:50	0.669	1.633	2.581	0.610	0.090
	1:100	0.685	1.521	1.638	0.669	0.054
	1:200	0.624	0.851	1.236	0.539	0.035
	1:400	0.466	0.547	0.977	0.403	0.022

Antigen 4 - Trypsin Inhibitor						
Antigen level	**Serum Dilution**	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5 (Normal mouse serum)**
10 μg/ml	1:50	0.154	0.105	0.247	0.065	0.060
	1:100	0.090	0.076	0.151	0.057	0.052
	1:200	0.067	0.047	0.085	0.048	0.034
	1:400	0.046	0.028	0.049	0.023	0.020
1 μg/ml	1:50	0.150	0.125	0.339	0.129	0.074
	1:100	0.097	0.083	0.233	0.084	0.051
	1:200	0.083	0.037	0.113	0.044	0.043
	1:400	0.040	0.031	0.077	0.032	0.024

Antigen 5 - Trypsinogen						
Antigen level	**Serum Dilution**	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5 (Normal mouse serum)**
10 μg/ml	1:50	0.077	0.117	2.580	2.051	0.083
	1:100	0.049	0.109	2.348	1.764	0.052
	1:200	0.036	0.073	1.925	1.249	0.036
	1:400	0.025	0.050	1.593	0.777	0.017
1 μg/ml	1:50	0.145	0.116	2.259	1.347	0.074
	1:100	0.071	0.069	2.010	1.271	0.050
	1:200	0.051	0.041	1.669	0.860	0.035
	1:400	0.030	0.024	1.307	0.545	0.023

Antigen 6 - Carbonic Anhydrase						
Antigen level	**Serum Dilution**	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5 (Normal mouse serum)**
10 μg/ml	1:50	0.135	0.137	3.069	2.870	0.097
	1:100	0.084	0.097	2.946	2.928	0.074
	1:200	0.043	0.047	2.919	2.827	0.049
	1:400	0.042	0.029	2.712	2.611	0.030
1 μg/ml	1:50	0.168	0.129	2.945	2.940	0.126
	1:100	0.075	0.097	2.811	2.760	0.078
	1:200	0.051	0.051	2.759	2.609	0.046
	1:400	0.031	0.031	2.551	2.146	0.029

Antigen 7 - Glyceraldehyde 3 Phosphate Dehydrogenase						
Antigen level	**Serum Dilution**	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5 (Normal mouse serum)**
10 μg/ml	1:50	0.117	0.078	0.238	0.127	0.239
	1:100	0.063	0.059	0.165	0.123	0.187
	1:200	0.063	0.037	0.112	0.053	0.143
	1:400	0.046	0.021	0.066	0.040	0.094
1 μg/ml	1:50	0.114	0.096	0.304	0.162	0.125
	1:100	0.081	0.065	0.227	0.111	0.102
	1:200	0.051	0.041	0.127	0.062	0.077
	1:400	0.031	0.023	0.086	0.043	0.043

Antigen 8 - Ovalbumin						
Antigen level	**Serum Dilution**	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5 (Normal mouse serum)**
10 μg/ml	1:50	1.442	1.065	1.371	1.258	0.101
	1:100	1.029	0.933	1.365	1.305	0.068
	1:200	0.779	0.714	1.074	1.169	0.044
	1:400	0.589	0.488	0.802	0.981	0.031
1 μg/ml	1:50	0.332	0.356	0.743	0.597	0.082
	1:100	0.256	0.240	0.681	0.531	0.062
	1:200	0.153	0.133	0.504	0.366	0.043
	1:400	0.086	0.065	0.355	0.242	0.040

Antigen 9 - Fumarase						
Antigen level	**Serum Dilution**	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5 (Normal mouse serum)**
10 μg/ml	1:50	2.690	2.620	2.734	2.813	0.187
	1:100	2.509	2.594	2.552	2.882	0.161
	1:200	2.486	2.222	2.186	2.674	0.133
	1:400	2.205	1.918	1.841	2.322	0.093
1 μg/ml	1:50	0.934	0.972	1.561	2.041	0.117
	1:100	0.871	0.908	1.652	2.150	0.097
	1:200	0.812	0.883	1.377	1.797	0.064
	1:400	0.737	0.703	1.031	1.394	0.032

Antigen 10 - Glutamic Dehydrogenase						
Antigen level	**Serum Dilution**	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5 (Normal mouse serum)**
10 μg/ml	1:50	0.111	0.086	3.214	3.150	0.296
	1:100	0.069	0.069	3.131	3.081	0.221
	1:200	0.071	0.044	3.123	2.788	0.159
	1:400	0.057	0.034	2.959	2.400	0.093
1 μg/ml	1:50	0.147	01.08	3.115	2.926	0.171
	1:100	0.087	0.097	2.858	2.722	0.123
	1:200	0.070	0.042	2.984	2.413	0.088
	1:400	0.057	0.021	2.815	1.984	0.050

Antigen 11 - Albumin						
Antigen level	Serum Dilution	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5 (Normal mouse serum)
10 μg/ml	1:50	0.295	0.213	1.251	0.202	0.064
	1:100	0.191	0.170	1.004	0.140	0.053
	1:200	0.116	0.091	0.576	0.081	0.030
	1:400	0.052	0.055	0.334	0.039	0.017
1 μg/ml	1:50	0.165	0.125	0.525	0.116	0.072
	1:100	0.120	0.091	0.257	0.087	0.057
	1:200	0.067	0.050	0.172	0.051	0.042
	1:400	0.051	0.024	0.709	0.037	0.026

Antigen 12 - Galactosidase						
Antigen level	Serum Dilution	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5 (Normal mouse serum)
10 μg/ml	1:50	2.735	2.480	2.965	3.060	0.671
	1:100	2.949	2.947	3.263	3.153	0.253
	1:200	2.852	2.905	2.998	3.218	0.168
	1:400	2.980	3.117	3.089	3.136	0.104
1 μg/ml	1:50	2.590	2.600	2.666	2.723	0.946
	1:100	2.632	2.656	2.816	2.836	0.651
	1:200	2.574	2.505	2.660	2.802	0.392
	1:400	2.526	1.997	2.685	2.726	0.199

TABLE 3

	Antigen											
Mouse	1	2	3	4	5	6	7	8	9	10	11	12
1	-	-	+++	-	-	-	-	+++	+++	-	+	+++
2	-	-	+++	-	-	-	-	+++	+++	-	+	+++
3	+	+	+++	+	+++	+++	-	+++	+++	+++	+++	+++
4	+	-	+++	-	+++	+++	-	+++	+++	+++	+	+++

The data presented in Table 3 emulate a more complex primary array that is employed to screen a far greater number of hybridoma supernatants and/or phage-derived antibody elements than exemplified herein. The only difference between the subject matter exemplified herein and such a large-scale screening is that the latter depends heavily upon automation and robotics to handle the larger volume of samples, and a greater reliance upon computing to handle data-processing.

A dotblot of mouse bleeds against these 12 immunising antigens was also performed. Antigens were dotted onto nitrocellulose, such that 10 ug/ of each antigen in 10 ug/ml was spotted onto 5 sheets of nitrocellulose in a grid pattern. The nitrocellulose sheets were dried for 2 hours at ambient temperature and blocked for 1 hour at ambient temperature, using 5% (w/v) Blotto. Mouse antisera obtained from each of immunised mice (mouse #1-mouse #4), and a non-immunised control (i.e. mouse #5) were diluted 1:400 in Blotto and incubated with the nitrocellulose sheets for 1 hour at room temperature. An individual sheet, containing each of the 12 antigens was used per mouse serum tested. Nitrocellulose sheets were then washed 3 times, for 5 minutes per wash, with PBS/tween solution. Sheep anti- mouse conjugate (AMRAD Silenus product code DAH), diluted 1:1000 in 5% (w/v) Blotto was added to the nitrocellulose sheets and incubated for 1 hour room temperature. The wash was repeated as before. Renaissance enhanced chemiluminscence substrate (New England Nuclear) was then

added and incubated for 2 minutes and the nitrocellulose sheets were then exposed to film for 2 minutes. Results are presented in Figure 6 and in Table 4.

The data obtained were normalised by subtracting the background (i.e. mouse #5) for each antigen in the mixture from the signal obtained for that antigen, in each test mouse. The normalised data are presented in Table 5.

TABLE 4

	Antigen											
Mouse	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	-	-	+++	+/-	-	+++	++	++++	++++	+/-	++++	+++
2	-	-	++++	-	-	+++	++	++++	++++	+/-	+++	+++
3	-	-	++++	+	+++	++++	++	++++	++++	+++	+++	+++
4	-	-	+++	-	+++	++++	++	++++	++++	+++	+++	+++
5	-	-	-	-	-	++	+	+/-	+/-	-	-	+

TABLE 5

	Antigen											
Mouse	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	-	-	+++	+/-	-	+	+	++++	++++	+/-	++++	+++
2	-	-	++++	-	-	+	+	++++	++++	+/-	+++	+++
3	-	-	++++	+	+++	++	+	++++	++++	+++	+++	+++
4	-	-	+++	-	+++	++	+	++++	++++	+++	+++	+++

A comparison of the data presented in Tables 3 and 5 indicate that most antigens perform similarly in ELISA and Dotblot, with the exception of antigen 11 (albumin), which was not highly reactive in ELISA for all mice with except mouse#3, but was highly reactive for all mice in dotblot assays.

These data demonstrate the possibility of immunising mice with a mixture of antigens and separating the polyclonal response into its monoclonal elements. Accordingly, these data provide enablement for separating the monoclonal specificities of a polyclonal response that is obtained when animals are simultaneously immunised with multiple antigens and hybridomas prepared therefrom which hybridomas express more than one monoclonal antibody. In particular, the primary protein array described

herein, or replicates thereof, are used to screen for individual monoclonal antibodies in a polyclonal mixture that can then be incorporated into secondary antibody arrays. The monoclonal elements of the immune response are exposed one at a time to identify monoclonal antibodies binding to different epitopes and/or monoclonal antibodies having different association/dissociation constants.

EXAMPLE 2

Construction of a secondary array

The primary array of 12 antigens described in the preceding Example, was screened with monoclonal antibodies prepared from the two highest-responding mice that had been immunised with a mixture of these 12 antigens (i.e. mouse #3 and mouse #4 described in Example 1).

More particularly, mice were boosted intraperitoneally with 15μg of the 12-antigen mixture in PBS, three days prior to fusion. On the day of fusion, spleens were removed from mouse #3 and mouse #4 (CBA), and a single cell suspension made from each. Spleen cells from each mouse were then centrifuged, together with 10^8 SP2/O myeloma cells. Fusions were performed by adding 1ml of PEG (1600) to each spleen cell/myeloma cell pellet, over a period of 1 min duration with gentle mixing, and the cell suspensions were then slowly diluted with DME, over a period of 10 minutes.

Fused cells were centrifuged gently and resuspended in Hybridoma Serum Free Medium (HSFM), containing 10% (v/v) Foetal Calf Serum (FCS), 10% (v/v) IL-6-conditioned medium and HAT medium, (i.e. H/10/10/HAT medium). Each fusion was plated out over approximately 12 microtitre plates (i.e.12 hybridoma plates), using 200μL of fusion mixture per microtitre well. Culture medium was changed 4 days and

8 days post-fusion, by removing the supernatants and adding fresh H/10/10/HAT medium. Supernatants were removed for assay by ELISA, 12 days post-fusion.

To perform primary ELISA screening of fusion cell culture supernatants derived from mouse number 3, six (6) ELISA plates (12 x 96 microtitre plates) were each coated with 50μl of each of the 12 individual antigens from the immunisation mixture referred to in the preceding Example (i.e. the primary array). Each antigen was diluted to 10μg/ml in carbonate buffer, pH 9.6, prior to coating. Plates were incubated for 20 hours at 4°C. The layout of the primary array is presented in Table 6.

TABLE 6

Antigen	Plate Number	Antigen	Plate Number
1	55-60	7	13-18
2	31-36	8	7-12
3	67-72	9	43-48
4	61-66	10	49-54
5	1-6	11	25-30
6	37-42	12	19-24

The primary array plates were blocked for 1 hour at room temperature with 50 μl 0.2% casein/PBS per well. Plates were washed three times using PBS/tween solution and hybridoma supernatants, diluted 1:2 with PBS, added to each well.

Each hybridoma was used to screen each antigen, by transferring 50 μl of each of diluted fusion cell culture supernatant from hybridoma plates 1-12 to antigen-coated plates 1-72 (i.e. supernatants from hybridoma plate 1 were transferred to antigen-coated plates 1, 7, 13, 19, 25, 31; supernatants from hybridoma plate 2 were transferred to antigen-coated plates 2, 8, 14, 20, 26, 32; supernatants from hybridoma plate 3 were transferred to antigen-coated plates 3, 9, 15, 21, 27, 33; supernatants

from hybridoma plate 4 were transferred to antigen-coated plates 4, 10, 16, 22, 28, 34; supernatants from hybridoma plate 5 were transferred to antigen-coated plates 5, 11, 17, 23, 29, 35; supernatants from hybridoma plate 6 were transferred to antigen-coated plates 6, 12, 18, 24, 30, 36; supernatants from hybridoma plate 7 were transferred to antigen-coated plates 37, 43, 49, 55, 61, 67; supernatants from hybridoma plate 8 were transferred to antigen-coated plates 38, 44, 50, 56, 62, 68; supernatants from hybridoma plate 9 were transferred to antigen-coated plates 39, 45, 51, 57, 63, 69; supernatants from hybridoma plate 10 were transferred to antigen-coated plates 40, 46, 52, 58, 64, 70; supernatants from hybridoma plate 11 were transferred to antigen-coated plates 41, 47, 53, 59, 65, 71; and supernatants from hybridoma plate 12 were transferred to antigen-coated plates 42, 48, 54, 60, 66, 72).

Plates 1-72 were incubated for 1 hour at 73°C in humidified box, washed three times using PBS/tween, and 50 μl of a 1:4,000 dilution of anti-mouse serum conjugated to HRP (Silenus product code DAH),in TBS/BSA/tween solution, was added to each well. The plates were then incubated for 1 hour at 37°C, washed three times as before in PBS/tween and 50 μl of OPD substrate was added to each well. P were incubated for 30 minutes at room temperature, and the absorbance at 492 nm, with a reference wavelength of 620 nm, was determined.

Results are presented in Table 7. Positive samples were retained for storage by freezing. Positive samples were defined as those having an absorbance value at 492 nm of 0.2 units, or greater. For all antigens except for antigen #1 (Aprotinin), it was possible to select positive hybridoma samples for storage producing absorbance values at 492 nm of 0.3 units or greater, based upon the higher antibody titre produced by those hybridomas. Only a few low-level reactor hybridomas were obtained to antigen #1, which were selected and are tested in a confirmatory screen.

Hybridomas selected for storage were transferred to 1ml microtitre wells in H/10/10/HAT medium. As cells became confluent, they were expanded to 6 ml wells

for freezing. Approximately 2-3x10^6 cells from 6 ml hybridoma cultures were collected into tubes, centrifuged, and the supernatants collected. The cells were resuspended in 1ml freezing medium (90% FCS, 10% DMSO), transferred to freezing vials, frozen slowly, and transferred to liquid nitrogen for long-term storage. Supernatants were stored frozen at -20°C until ready for use.

Hybridoma cell culture supernatants from approximately 100 positive hybridomas obtained in the primary screening were re-screened by ELISA using similar conditions as states *supra*, except that supernatants were used undiluted and each supernatant was screened against each antigen.

To construct the primary array, twelve plates, numbered 1-12, were coated with antigen numbers 1-12.

Additionally, a further two plates, numbered plate # 13 and plate #13, were coated as follows:

Plate #13: column 1; antigen 5; column 3, antigen 8; column 5, antigen 7; column 7, antigen 12; column 9, antigen 11; and column 11, antigen 2.

Plate #14: column 1; antigen 6; column 3, antigen 9; column 5, antigen 10; column 7, antigen 1; column 9, antigen 4; and column 11, antigen 3.

The layout of hybridoma supernatants on each of plates 1-12 is shown in Table 8. The layout of hybridoma supernatants on each of plates 13 and 14 is shown in Table 9.

Data presented in Table 10 are representative of the secondary array of the invention and exemplify the ability of the present invention to produce a fingerprint or unique tag to each of the 12 target antigens tested, through the recognition patterns of one or more antibodies at greater than two standard deviations above a background response (i.e. a positive response). The data provided herein indicate the general applicability

of the invention to producing a fingerprint or unique tag to the antigens in a highly complex cellular extract, wherein larger primary and secondary arrays, such as miniaturised arrays, are employed, together with highly-sensitive detection systems.

TABLE 7

PLATES 1 - 6							
Plate	**Position**	**Antigen 5**	**Antigen 8**	**Antigen 7**	**Antigen 12**	**Antigen 11**	**Antigen 2**
1	1A1	0.013	0.011	0.014	0.024	0.019	**1.069**
	1A2	0.068	**0.205**	0.164	0.118	**0.229**	0.419
	1E5	0.007	0.006	0.048	0.01	0.019	**1.341**
2	2C2	**0.216**	0.184	**0.333**	0.024	0.06	0.029
	2B3	**0.365**	0.124	0.172	0.023	0.019	0.053
	2F5	0.027	**0.483**	**0.383**	0.017	0.046	0.091
	2H7	0.015	**0.335**	0.011	0.007	0.033	0.042
	2D10	**1.663**	**1.676**	**1.366**	**1.56**	**1.466**	**1.652**
3	3E1	0.008	0.017	0.052	0.013	**0.382**	**0.314**
	3H3	0.078	0.023	0.097	0.045	0.077	**1.113**
	3D4	0.027	0.036	0.059	0.041	**1.152**	0.177
	3D6	0.012	0.017	0.084	0.012	**2.631**	0.14
	3E7	**0.76**	0.01	0.058	0.011	0.06	0.07
	3G6	0.018	0.029	0.098	**0.209**	0.125	0.037

	3H8	0.014	0.021	0.067	0.016	**1.126**	0.087
	3D10	0.021	0.058	**0.274**	0.035	0.47	0.05
	3B12	**0.279**	0.081	**0.583**	0.129	**0.398**	**0.281**
4	4B2	0.024	0.026	0.024	0.022	0.034	**2.071**
	4E4	**0.241**	0.092	**0.402**	0.037	**1.637**	0.112
	4G4	0.026	0.027	0.022	0.013	0.135	0.043
	4A7	**0.792**	0.033	**1.038**	0.098	0.076	0.02
	4B7	0.023	0.029	**0.719**	0.028	0.133	0.019
	4E7	0.063	0.01	**0.414**	0.03	0.039	0.032
	4F7	0.016	0.063	**0.627**	0.053	0.055	0.024
	4G7	0.02	**0.505**	0.068	0.04	0.064	0.021
	4H7	0.058	0.033	0.021	**0.311**	0.032	**0.686**
	4H8	0.077	**0.402**	0.012	0.018	0.101	0.024
	4G9	0.103	**0.314**	0.113	0.04	0.127	0.033
	4B11	0.063	0.023	0.097	**0.437**	0.05	0.019
	4F11	0.038	0.028	0.129	0.01	**0.409**	0.096
	4B12	**0.708**	0.016	**0.75**	0.044	0.05	0.017
	4C12	**0.367**	0.02	0.061	0.026	0.023	**0.682**

5	5G1	0.017	0.022	0.022	**0.885**	0.137	0.05
	5D3	0.025	0.022	0.071	0.193	0.02	**1.446**
	5F3	0.028	0.017	0.016	**0.249**	0.022	**0.577**
	5C4	0.018	0.011	0.023	**0.202**	**1.741**	0.078
	5G4	0.029	0.031	0.017	0.191	0.037	**1.443**
	5E6	0.018	0.009	0.076	**0.285**	0.04	**0.896**
	5F6	0.018	0.026	0.02	**0.441**	0.039	**0.231**
	5A7	0.014	**0.678**	0.015	0.151	0.027	0.026
	5E7	0.018	0.033	0.058	**0.417**	0.07	**0.942**
	5F7	0.017	0.029	0.021	**0.373**	0.082	**2.052**
	5F8	0.019	**2.315**	0.034	**0.248**	0.028	**0.832**
	5G10	0.068	**0.801**	0.008	**0.236**	0.04	0.061
6	6C1	**1.266**	0.027	**0.363**	0.032	0.026	0.024
	6B3	**0.379**	0.182	0.112	**0.207**	**0.352**	0.329
	6C6	**0.434**	0.045	0.017	0.012	0.053	0.027
	6G7	0.053	0.077	0.237	0.067	**0.303**	**0.498**
	6A9	0.014	0.016	0.019	0.051	**2.189**	0.017
	6D9	0.091	0.08	**0.659**	0.017	0.048	0.042

	6F9	0.022	0.031	**0.483**	0.021	0.037	0.03
	6H9	0.014	0.036	0.028	**0.85**	0.098	0.029
	6A11	**0.325**	0.027	0.014	0.098	0.027	0.02
	6F12	0.015	**0.719**	0.016	0.011	**0.507**	0.037
	6G12	**1.999**	0.056	0.068	0.015	0.047	0.026
Total pos. [A]		11	10	13	8	14	18
Total pos. [B]		2	1	2	7	1	2

PLATES 7 - 12							
Plate	**Position**	**Antigen 6**	**Antigen 9**	**Antigen 10**	**Antigen 1**	**Antigen 4**	**Antigen 3**
7	F4	0.1	0.153	**0.455**	**0.212**	**0.237**	0.168
	7F7	**0.28**	0.11	**0.344**	0.129	0.151	0.182
	7F9	0.032	0.015	0.025	0.016	**0.929**	0.016

	7F10	0.033	0.025	0.017	0.017	**0.664**	0.027
	7C11	0.02	0.012	0.008	0.019	0.014	**0.438**
	7E11	0.031	0.009	0.014	0.018	**0.223**	0.018
	7E12	0.131	0.06	**0.437**	0.054	0.079	0.105
8	8C1	0.027	0.049	0.032	**0.235**	0.031	0.033
	8G2	0.123	0.14	**0.926**	0.111	0.086	0.177
	8D4	**1.948**	0.01	0.029	0.013	0.038	0.041
	8G4	**0.626**	0.129	**0.894**	0.151	0.162	**0.259**
	8E5	0.114	0.01	**0.361**	0.019	0.015	0.046
	8D10	**0.33**	**0.323**	**0.687**	**0.264**	**0.228**	**0.29**
	8F10	**2.107**	0.012	0.029	0.023	0.023	0.039
9	9F1	**0.302**	0.034	0.081	0.051	0.056	0.06
	9F2	**0.418**	0.023	0.051	0.016	0.016	0.056
	9B3	**0.392**	0.054	0.043	0.012	0.022	0.059
	9D3	**0.846**	0.015	0.032	0.017	0.02	0.059
	9A4	0.03	0.023	0.032	0.006	**2.419**	0.059
	9G9	0.047	0.013	0.03	0.019	0.016	**0.521**

10	10H2	**0.727**	0.082	**0.256**	0.037	0.028	0.02
	10C3	0.076	**0.444**	0.172	0.033	0.023	0.028
	10H3	**0.893**	**0.262**	**0.579**	**0.215**	0.162	0.027
	10H4	**0.24**	0.175	**2.175**	0.115	0.093	0.031
	10F5	0.027	0.035	0.047	0.047	**0.377**	0.021
	10H7	**0.405**	0.052	0.052	0.052	0.042	0.021
	10H8	0.126	0.071	0.081	0.074	0.177	**0.301**
	10C10	0.061	**0.841**	**0.225**	0.147	0.053	0.028
	10D10	0.025	0.151	**0.811**	0.09	0.077	0.035
	10E10	0.039	0.073	**1.623**	0.108	0.065	0.116
	10F10	0.036	0.076	**0.362**	0.127	**0.562**	0.028
	10B11	0.086	**0.442**	**0.25**	0.141	0.148	0.071
	10B12	0.132	**0.361**	0.142	0.176	0.147	0.033
	10H12	0.136	**0.345**	**0.306**	0.128	0.076	0.024
11	11E7	0.136	0.024	0.051	0.028	0.051	**1.288**
	11D8	0.077	**0.367**	0.045	0.023	0.169	0.062
	11E8	0.135	**0.285**	0.051	0.062	0.071	**0.943**

	11F8	0.156	**0.255**	0.071	0.054	0.086	**0.241**
	11C10	0.148	**0.478**	0.054	0.026	0.066	0.126
	11D10	0.079	0.105	0.08	0.035	**0.239**	0.089
	11G11	0.118	0.093	0.075	0.076	0.071	**1.047**
	11H11	0.06	0.021	0.064	0.041	**0.238**	**0.345**
	11A12	**0.218**	0.101	0.097	0.053	0.059	**0.462**
	11B12	0.138	0.054	0.076	0.144	0.139	**0.944**
	11H12	0.11	0.041	0.122	0.072	0.112	**0.801**
12	12G4	0.081	0.033	0.056	0.032	0.037	**0.267**
	12H8	0.103	0.024	0.059	0.025	0.227	0.065
Total pos.[A]		11	8	13	0	5	10
Total pos.[B]		2	3	3	4	6	2

A, positive values having OD>0.3.

B, positive values 0.2>OD<0.3.

TABLE 8

	1	2	3	4	5	6	7	8	9	10	11	12
A	1A1	1A2	1E5	2C2	2B3	2F5	2H7	2D10	3E1	3H3	3D4	3D6
B	3E7	3G8	3H8	3D10	3B12	4B12	4F7	4G7	4H8	4G9	4B11	4F11
C	5F6	5A7	5E7	5F7	5F8	5G10	6C1	6B3	6C6	6G7	6A9	6D9
D	6F9	6H9	6A11	6F12	6G12	7F4	7F7	7F9	7F10	7C11	7E11	7E12
E	8C1	8G2	8D4	8G4	8E5	8H8	8G9	8D10	8F10	9F2	9B3	9D3
F	9H3	9A4	9H4	5G5	9D10	9E10	10C3	10H3	10H4	10F5	10F7	10H8
G	10C10	10D10	10E10	10F10	10B11	10B12	10H12	11H3	11E7	11D8	11E8	11F8
H	11C10	11D10	11G11	11H11	4B12	4C12	5G1	5D3	5F3	5G4	5C4	5E6

TABLE 9

	1	2	3	4	5	6	7	8	9	10	11	12
A	11A12		11A12		11A12		11A12		11A12		11A12	
B	11F12		11F12		11F12		11F12		11F12		11F12	
C	11H12		11H12		11H12		11H12		11H12		11H12	
D	12A12		12A12		12A12		12A12		12A12		12A12	
E	12G4		12G4		12G4		12G4		12G4		12G4	
F	12H8		12H8		12H8		12H8		12H8		12H8	
G	4G7		4G7		4G7		4G7		4G7		4G7	
H												

TABLE 10

Plate	Clone	ANTIGEN NUMBER											
		5	8	7	12	11	2	6	9	10	1	4	3
1	1A1	.014	.021	.022	2.115	.012	.012	.02	.251	.031	.045	.028	.035
	1A2	.024	.062	.031	.174	.034	.121	.059	.228	.069	.001	.065	.069
	1E5	.009	.022	.037	.043	.02	.013	.018	.144	.026	.073	.042	.028
2	2C2	.014	.031	.026	.033	.023	.017	.024	.197	.055	.053	.053	.039
	2B3	.006	0.16	.022	.033	.003	.006	.012	.165	0026	.036	.016	.024
	2F5	.015	.022	.034	.044	.007	.015	.02	.131	.043	.031	.018	.025
	2H7	.011	.018	.02	.034	.007	.011	.014	.152	.023	.028	.022	.019
	2D10	1.661	1.545	1.896	1.813	1.473	1.327	1.828	1.844	2.077	1.916	1.639	1.656
3	3E1	.013	.016	.024	.024	.005	.007	.01	.376	.025	.034	.013	.021
	3H3	.036	.047	.043	.154	.071	.18	.107	.156	.033	.049	.038	.02
	3D4	.134	.092	.205	.205	.125	.17	.318	.608	.378	.134	.08	.141
	3D6	.018	.02	.033	.033	.012	.005	.015	2.238	.021	.058	.058	.048
	3E7	.016	.212	.135	.086	.019	.014	.088	.242	.089	1.108	.043	.028
	3G8	.029	.032	.031	.019	.013	.009	.016	.296	.025	.026	.042	.026
	3H8	.022	.016	.028	.031	.014	.012	.029	.244	.033	.021	.044	.025

	3D10	.088	.024	.013	.016	.008	.009	.011	.17	.018	.037	.049	.027
	3B12	.098	**.273**	.093	**.480**	**.264**	**.336**	**.586**	**.372**	.175	.121	**.442**	.128
4	4B2	.011	.018	.02	**2.017**	.008	.008	.012	.167	.013	.016	.017	.02
	4F7	.011	.076	.021	.025	.015	.008	.017	.133	.023	.038	.019	.038
	4G7	.032	.02	.033	.034	.011	.008	.018	.02	.028	.012	.022	.015
	4H7	.013	.017	.069	.151	.01	.008	.032	.149	.039	**1.021**	.019	.036
	4H8	.002	.017	.023	.021	.006	.007	.017	.168	.015	.015	.021	.02
	4G9	.025	.094	.048	.142	.063	.094	.123	.205	.084	.128	.144	.045
	4B11	.011	.016	.024	.023	.01	.009	.027	.119	.019	.02	.019	.034
	4F11	.009	.022	**.204**	.027	.014	.014	.032	.141	.169	.023	.023	.096
	4B12	.004	.009	.019	.02	.01	.011	.022	.045	.017	.026	.034	.026
	4C12	.005	.132	.009	.102	.012	.009	.023	.027	.011	.023	.019	.022
5	5G1	.009	.011	.014	.015	.015	.014	.028	.037	.012	.022	.019	.022
	5D3	.010	.027	.016	.027	.02	.014	.033	.054	.022	.035	.033	.028
	5F3	.003	.020	.018	.024	.02	.011	.016	.096	.014	.027	.023	.019
	5C4	.017	.021	.038	.07	.024	.026	.052	.073	.05	.045	.029	.052
	5G4	.050	.053	.055	**.445**	.075	**.219**	**.272**	.122	**.224**	.071	.067	.095
	5E6	.006	.019	.013	.017	.013	.015	.03	.117	.017	.046	.025	.037

	5F6	.022	.024	.071	.045	.016	.041	.038	.220	.042	.031	.037	.033
	5A7	.013	.022	.028	.032	.012	.01	.021	.225	.025	.025	.036	.044
	5E7	.011	.016	.013	.016	.013	.008	.008	.181	.01	.014	.04	.016
	5F7	.036	.024	.077	.115	.026	.04	.067	.184	.145	.042	.042	.266
	5F8	.005	.015	.015	.032	.033	.016	.017	.175	.019	.014	.03	1.415
	5G10	.013	.023	.016	.089	.019	.007	.012	.118	.014	.013	.026	.021
6	6C1	.006	.010	.014	.018	.015	.01	.013	.149	.022	.643	.03	.023
	6B3	.125	.26	.087	.430	.249	.364	.425	.336	.151	.180	.369	.181
	6C6	.004	.009	.007	.012	.005	.003	.008	.152	.003	.009	.021	.013
	6G7	.537	.336	.414	.992	.354	.449	.842	.704	.502	.312	.408	.392
	6A9	.006	.011	.026	.021	.008	.007	.019	.146	.011	.015	.015	.019
	6D9	.009	.027	.017	.199	.024	.016	.032	.144	.038	.045	.034	.039
	6F9	.017	.024	.013	.042	.08	.011	.021	.216	.032	.027	.031	.032
	6H9	.008	.012	.024	.033	.011	.013	.014	.231	.024	.054	.043	.044
	6A11	.011	.018	.018	.027	.011	.01	.017	.168	.017	.016	.033	.018
	6F12	.009	.013	.025	.018	.013	0.23	.01	.134	.031	.023	.038	.038
	6G12	.008	.01	.01	.021	.007	.007	.015	.152	.01	.016	.031	.031

7	7F4	.093	.098	.074	.258	.106	.183	.236	.219	.240	.092	.107	.121
	7F7	.155	.127	.249	.400	.19	.184	.402	.201	.325	.058	.203	.125
	7F9	.007	.01	.015	.019	.007	.006	.012	.095	.013	.015	.02	.017
	7F10	.003	.013	.021	.023	.009	.007	.052	.127	.028	.016	.031	.018
	7C11	.004	.011	.012	.015	.008	.007	.011	.122	.01	.015	.026	.014
	7E11	.006	.012	.013	.023	.008	.01	.014	.123	.015	.02	.017	.018
	7E12	.401	.253	.121	1.883	.481	.428	.596	.441	.632	.173	.278	.307
8	8C1	.012	.026	.013	.034	.014	.014	.017	.199	.021	.04	.031	.022
	8G2	.141	.143	.15	.648	.115	.229	.405	.292	.387	.103	.176	.125
	8D4	.008	.014	2.149	.013	.006	.005	.01	.127	.012	.018	.027	.015
	8G4	.369	.258	.249	.584	.497	.417	.576	.602	.510	.389	.313	.385
	8E5	.004	.008	.116	.028	.008	.004	.022	.109	.461	.019	.031	.02
	8D10	.230	.458	.145	.590	.452	.525	.585	.466	.481	.361	.647	.372
	8F10	.002	.014	1.474	.016	.009	.006	.032	.072	.055	.021	.026	.021
9	9F2	.003	.007	.006	.012	.008	.042	.023	.116	.009	.011	.017	.012
	9B3	.009	.004	.683	.012	.008	.009	.012	.102	.012	.019	.021	.018
	9D3	.01	.014	1.440	.02	.015	.019	.024	.087	.033	.032	.021	.039
	9A4	.015	.027	.027	.044	.013	.033	.026	.136	.016	.017	.037	.035

	9G9	.012	.031	.022	.016	.008	.007	.012	.109	.013	.018	.051	.029
10	10C3	.011	.023	.028	.035	.013	.05	.028	.012	.024	.02	.041	.03
	10H3	**.713**	**.490**	**.884**	**1.035**	**.746**	**.506**	**.870**	**.886**	**.948**	**.705**	**.567**	**.596**
	10H4	.071	.11	.123	**.219**	**.289**	.08	**.251**	**.243**	**2.006**	.043	.129	.064
	10F5	.004	.01	.012	.015	.005	.006	.008	.071	.01	.02	.036	.016
	10H7	.012	.023	.001	.025	.01	.018	.018	.127	.017	.027	.033	.019
	10H8	.007	.043	.017	.016	.017	.01	.026	.058	.027	.03	.024	.031
	10C10	.011	.018	.029	.041	.018	.017	.031	.096	.032	.042	.062	.034
	10D10	.014	.048	.021	.022	.011	.018	.034	.152	.023	.026	.036	.02
	10E10	.006	.006	.016	.013	.007	.011	.02	.103	.008	.028	.032	.021
	10F10	.011	.019	.018	.022	.009	.012	.017	.093	.017	.031	.039	.097
	10B11	.135	.149	.044	.192	.16	.135	.16	**.283**	.117	.176	**.222**	.194
	10B12	.023	.027	.161	.065	.022	.025	.037	.053	.06	.032	.069	.03
	10H12	.016	.02	.023	.060	.018	.026	.04	.065	.112	.032	.036	.046
11	11E7	.008	.02	.019	.023	.077	.009	.015	.087	.03	.023	.036	.027
	11D8	.005	.012	.024	.012	.011	.006	.019	.038	.017	.023	.03	.018
	11E8	.005	.011	.014	.033	.01	.01	.012	.069	.019	.025	.028	.023
	11F8	.009	.04	.019	.025	.022	.018	.038	.068	.023	.034	.022	.042

	11C10	.009	.012	.023	.035	.016	.021	.025	.168	.035	.031	.038	.026
	11D10	.006	.021	.017	.035	.02	.02	.025	.172	.008	.042	.018	.023
	11G11	.008	.031	.018	.026	.014	.019	.028	.135	.011	.031	.032	.028
	11H11	.009	.03	.024	.049	.028	.029	.029	.098	.023	.03	.034	.039
	11A12	.011	.041	.03	.018	.01	.01	.012	.019	.015	.019	.028	.018
	11F12	.035	.024	.115	.048	.017	.017	.045	.021	.049	.016	.026	.016
	11H12	.010	.022	.147	.022	.033	.005	.009	.009	.01	.009	.025	.003
12	12G4	.057	.210	.067	.186	.051	.051	.106	.081	.104	.044	.09	.05
	12H8	.009	.017	.014	.01	.006	.009	.018	.013	.011	.007	.009	.006
Total Pos. OD>0.3		5	4	7	13	6	8	11	11	11	8	7	7
Total Pos. OD<0.3		1	6	4	3	3	2	3	15	2	0	3	1

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CLAIMS:

1. A method of determining the protein profile of a biological sample comprising:

(i) preparing a primary array of proteins $a^1_{(Xn,Yn)}$, $a^2_{(Xn,Yn)}$, $a^3_{(Xn,Yn)}$,.....,$a^n_{(Xn,Yn)}$ comprising a multiplicity of protein elements designed to emulate a significant portion of the antigenic diversity of a cell, tissue, organ, organism from which the biological sample is derived, wherein $a^1,a^2,a^3,....,a^n$ are proteins; Xn is the coordinate of any particular protein along a first dimension of said array; Yn is the coordinate of any particular protein along a second dimension of said array; and wherein n is any positive finite number;

(ii) screening the primary array with a plurality of monoclonal antibodies and/or antibody variants and/or derivatives one-at-a-time, or a reduced pool thereof one-at-a-time, so as to determine those antibodies and/or antibody variants or derivatives that bind to one or more proteins in said primary array;

(iii) preparing a secondary array of monoclonal antibodies and/or antibody variants or derivatives $Ab^1_{(Xn,Yn)}$, $Ab^2_{(Xn,Yn)}$, $Ab^3_{(Xn,Yn)}$,.....,$Ab^n_{(Xn,Yn)}$, wherein $Ab^1,Ab^2,Ab^3,....,Ab^n$ are monoclonal antibodies and/or antibody variants or derivatives that bind specifically or non-specifically to one or more proteins in the primary array; Xn is the coordinate of any particular monoclonal antibody and/or antibody variant or derivative along a first dimension of said array; Yn is the coordinate of any particular monoclonal antibody and/or antibody variant or derivative along a second dimension of said array; and wherein n is any positive finite number; and

(iv) screening the secondary array with said biological sample to determine those proteins in said biological sample which bind to one or more monoclonal antibodies and/or antibody variants or derivatives in said secondary array in a uniquely-definable manner.

2. The method according to claim 1, further comprising the step of identifying one or more proteins in the primary array which form antigen-antibody complexes with

elements of the secondary array as determined at (iv), wherein said step comprises identifying the coordinates (Xn,Yn) of one or more proteins in the primary array which bind to one or more monoclonal antibodies and/or antibody variants or derivatives in the secondary array.

3. The method according to claims 1 or 2 further comprising the step of isolating one or more proteins from the biological sample which bind to one or more monoclonal antibodies and/or antibody variants or derivatives in the secondary array.

4. The method according to claims 2 or 3, further comprising the step of determining the amino acid sequence and/or post-translational modifications of the isolated protein and/or of the isolated protein and/or one or more proteins identified in the primary array.

5. The method according to claim 4, further comprising isolating a DNA molecule encoding the isolated protein and/or one or more proteins identified in the primary array and expressing said DNA to produce one or more recombinant proteins identified in the primary array.

6. The method according to any one of claims 1 to 5, wherein the primary array comprises elements derived from one or more complex mixtures of proteins resolved by two-dimensional gel electrophoresis.

7. The method according to any one of claims 1 to 6, wherein the primary array and/or the secondary array is/are bound to a solid porous or non-porous support or matrix prior to being screened.

8. The method according to claim 7, wherein the solid porous or non-porous support or matrix comprises a polyvinylidene difluoride (PVDF) membrane, nitrocellulose membrane, nylon membrane or other porous or non-porous membrane

to which the proteins of the primary array and/or the monoclonal antibodies and/or antibody variants or derivatives of the secondary array are transferred.

9. The method according to claim 7, wherein the solid support or matrix comprises a plurality of polymeric pins.

10. The method according to claim 7, wherein the solid support or matrix comprises a plurality of microtitre wells.

11. The method according to claim 7, wherein the solid support or matrix comprises one or more silicon chips.

12. The method according to any one of claims 1 to 11, wherein the monoclonal antibody and/or antibody variant or derivative that is used to screen the primary array is derived from a hybridoma or other cell which expresses antibodies which bind to one or more proteins in said primary array.

13. The method according to any one of claims 1 to 11, wherein the antibody variant or derivative that is used to screen the primary array is derived from a bacteriophage or virus particle which expresses antibodies that bind to one or more proteins in said primary array.

14. The method according to any one of claims 1 to 13, wherein the proteins of the primary array are synthetic peptides, synthetic oligopeptides or synthetic polypeptides.

15. The method according to any one of claims 1 to 13, wherein the proteins of the primary array are recombinant peptides, recombinant oligopeptides or recombinant polypeptides.

16. The method according to claims 14 or 15, wherein the synthetic or recombinant

peptides, oligopeptides or polypeptides are derived from one or more peptide libraries, and/or induced peptide expression libraries and/or protein expressed from within one or more cloned gene libraries.

17. The method according to any one of claims 1 to 13, wherein the proteins of the primary array are naturally-occurring peptides, oligopeptides, polypeptides, proteins or enzymes.

18. The method according to any one of claims 1 to 17, wherein the proteins in the biological sample used to screen the secondary array are labelled with one or more reporter molecules prior to screening said secondary array therewith.

19. The method according to claim 18, wherein the reporter molecule comprises a FLAG peptide, poly-His amino acid sequence or poly-Lys amino acid sequence or other known amino acid string.

20. The method according to claim 18, wherein the reporter molecule comprises an isotope, fluorescent or enzymatic tag.

21. The method according to any one of claims 1 to 20, wherein the screening of the primary array and/or the screening of the secondary array is normalised to reduce or remove concentration-dependent variation and/or antigen-specific variation in signal intensity.

22. The method according to claim 21, wherein the screening of the primary array is normalised to reduce or remove concentration-dependent variation, said normalisation comprising:

(i) labelling the proteins of said array with one or more reporter molecules;

(ii) screening the labelled primary array with an antibody against the reporter molecule;

(iii) determining both the signal obtained using the antibody against the reporter molecule and the signal obtained using the monoclonal antibody and/or antibody variant or derivative; and

(iv) adjusting the signal intensity obtained using the monoclonal antibody and/or antibody variant or derivative to account for the concentration of protein as determined by the signal intensity obtained using the antibody against the reporter molecule.

23. The method according to claim 22, wherein the reporter molecule is an immunogenic peptide or protein region or other immunogenic amino acid sequence.

24. The method according to claim 23, wherein the immunogenic peptide or protein region or other immunogenic amino acid sequence is fused to the proteins of the primary array.

25. The method according to claims 23 or 24, wherein the immunogenic peptide or protein region or other immunogenic amino acid sequence comprises a FLAG peptide, poly-His amino acid sequence or poly-Lys amino acid sequence or other known amino acid string.

26. The method according to claim 21, wherein the screening of the primary array is normalised to reduce or remove antigen-specific variation, said normalisation comprising averaging the signal intensity obtained using monoclonal antibodies and/or antibody variants or derivatives that bind to different epitopes on the same protein.

27. The method according to claim 21, wherein the screening of the secondary array is normalised to reduce or remove concentration-dependent variation, said normalisation comprising:

(i) screening the secondary array with one or more reporter molecules that bind to all monoclonal antibodies and/or antibody variants or derivatives in said

array;

(iii) determining both the signal obtained using the reporter molecule and the signal obtained using the biological sample; and

(iv) adjusting the signal intensity obtained using the biological sample to account for the concentration of protein as determined by the signal intensity obtained using the reporter molecule.

28. The method according to claim 27, wherein the reporter molecule comprises protein A, a lectin or a secondary antibody that binds to the monoclonal antibodies and/or antibody variants or derivatives of the secondary array.

29. A method of determining one or more proteins that are differentially-expressed between cells, tissues, organs, or organisms or biological samples derived therefrom comprising:

(i) preparing a primary array of proteins $a^1_{(Xn,Yn)}$, $a^2_{(Xn,Yn)}$, $a^3_{(Xn,Yn)}$,.....,$a^n_{(Xn,Yn)}$ comprising a multiplicity of protein elements designed to emulate a significant portion of the antigenic diversity of a cell, tissue, organ, organism from which the biological sample is derived, wherein a^1,a^2,a^3,.....,a^n are proteins; Xn is the coordinate of any particular protein along a first dimension of said array; Yn is the coordinate of any particular protein along a second dimension of said array; and wherein n is any positive finite number;

(ii) screening the primary array with a plurality of monoclonal antibodies and/or antibody variants and/or derivatives one-at-a-time, or a reduced pool thereof one-at-a-time, so as to determine those antibodies and/or antibody variants or derivatives that bind to one or more proteins in said primary array;

(iii) preparing a secondary array of monoclonal antibodies and/or antibody variants or derivatives $Ab^1_{(Xn,Yn)}$, $Ab^2_{(Xn,Yn)}$, $Ab^3_{(Xn,Yn)}$,......,$Ab^n_{(Xn,Yn)}$, wherein Ab^1,Ab^2,Ab^3,.....,Ab^n are monoclonal antibodies and/or antibody variants or derivatives that bind specifically or non-specifically to one or more proteins in the primary array; Xn is the coordinate of any particular monoclonal antibody

and/or antibody variant or derivative along a first dimension of said array; Yn is the coordinate of any particular monoclonal antibody and/or antibody variant or derivative along a second dimension of said array; and wherein n is any positive finite number; and

(iv) separately screening the secondary array with two or more biological samples derived from said cells, tissues, organs, or organisms, and comparing the signals obtained using each of said biological samples to determine those proteins which are differentially expressed.

30. The method according to claim 29, further comprising the step of identifying one or more proteins in the primary array which form antigen-antibody complexes with elements of the secondary array as determined at (iv), wherein said step comprises identifying the coordinates (Xn,Yn) of one or more proteins in the primary array which bind to one or more monoclonal antibodies and/or antibody variants or derivatives in the secondary array.

31. The method according to claims 29 or 30 further comprising the step of isolating one or more of the differentially-expressed proteins.

32. The method according to any one of claims 29 to 31 further comprising the step of determining the amino acid sequence and/or post-translational modifications of the isolated protein and/or of the isolated protein and/or one or more proteins identified in the primary array.

33. The method according to claim 32, further comprising isolating a DNA molecule encoding the isolated protein and/or one or more proteins identified in the primary array and expressing said DNA to produce one or more recombinant proteins identified in the primary array.

34. The method according to any one of claims 29 to 33, wherein the array

comprises elements derived from one or more complex mixtures of proteins resolved by two-dimensional gel electrophoresis.

35. The method according to any one of claims 29 to 34, wherein the primary array and/or the secondary array is/are bound to a solid porous or non-porous support or matrix prior to being screened.

36. The method according to claim 35, wherein the solid porous or non-porous support or matrix comprises a polyvinylidene difluoride (PVDF) membrane, nitrocellulose membrane, nylon membrane or other porous or non-porous membrane to which the proteins of the primary array and/or the monoclonal antibodies and/or antibody variants or derivatives of the secondary array are transferred.

37. The method according to claim 35, wherein the solid support or matrix comprises one or more silicon chips.

38. The method according to claim 35, wherein the solid support or matrix comprises a plurality of microtitre wells.

39. The method according to claim 35, wherein the solid support or matrix comprises a plurality of polymeric pins.

40. The method according to any one of claims 29 to 39, wherein the monoclonal antibody and/or antibody variant or derivative that is used to screen the primary array is derived from a hybridoma or other cell which expresses antibodies which bind to one or more proteins in said primary array.

41. The method according to any one of claims 29 to 39, wherein the antibody variant or derivative that is used to screen the primary array is derived from a bacteriophage or virus particle which expresses antibodies that bind to one or more

proteins in said primary array.

42. The method according to any one of claims 29 to 41, wherein the proteins of the primary array are synthetic peptides, synthetic oligopeptides or synthetic polypeptides.

43. The method according to any one of claims 29 to 41, wherein the proteins of the primary array are recombinant peptides, recombinant oligopeptides or recombinant polypeptides.

44. The method according to claims 42 or 43, wherein the synthetic or recombinant peptides, oligopeptides or polypeptides are derived from one or more peptide libraries, and/or induced peptide expression libraries and/or protein expressed from within one or more cloned gene libraries.

45. The method according to any one of claims 29 to 41, wherein the proteins of the primary array are naturally-occurring peptides, oligopeptides, polypeptides, proteins or enzymes.

46. The method according to any one of claims 29 to 45, wherein the proteins in the biological sample used to screen the secondary array are labelled with one or more reporter molecules prior to screening said secondary array therewith.

47. The method according to claim 46, wherein the reporter molecule comprises a FLAG peptide, poly-His amino acid sequence or poly-Lys amino acid sequence or other known amino acid string.

48. The method according to claim 46, wherein the reporter molecule comprises a radioactive isotope, fluorescent or enzymatic tag.

49. The method according to any one of claims 29 to 48, wherein the screening of

the primary array and/or the screening of the secondary array is normalised to reduce or remove concentration-dependent variation in signal intensity.

50. The method according to claim 49, wherein the screening of the primary array is normalised to reduce or remove concentration-dependent variation, said normalisation comprising:

(i) labelling the proteins of said array with one or more reporter molecules;

(ii) screening the labelled primary array with an antibody against the reporter molecule;

(iii) determining both the signal obtained using the antibody against the reporter molecule and the signal obtained using the monoclonal antibody and/or antibody variant or derivative; and

(iv) adjusting the signal intensity obtained using the monoclonal antibody and/or antibody variant or derivative to account for the concentration of protein as determined by the signal intensity obtained using the antibody against the reporter molecule.

51. The method according to claim 50, wherein the reporter molecule is an immunogenic peptide or protein region or other immunogenic amino acid sequence.

52. The method according to claim 51, wherein the immunogenic peptide or protein region or other immunogenic amino acid sequence is fused to the proteins of the primary array.

53. The method according to claims 51 or 52, wherein the immunogenic peptide or protein region or other immunogenic amino acid sequence comprises a FLAG peptide, poly-His amino acid sequence or poly-Lys amino acid sequence or other known amino acid string.

54. The method according to claim 49, wherein the screening of the secondary

array is normalised to reduce or remove concentration-dependent variation, said normalisation comprising:

(i) screening the secondary array with one or more reporter molecules that bind to all monoclonal antibodies and/or antibody variants or derivatives in said array;

(iii) determining both the signal obtained using the reporter molecule and the signal obtained using one or more of the biological samples that produce a positive signal; and

(iv) adjusting the signal intensity obtained using said one or more biological samples to account for the concentration of protein as determined by the signal intensity obtained using the reporter molecule.

55. The method according to claim 54, wherein the reporter molecule comprises protein A, a lectin or a secondary antibody that binds to the monoclonal antibodies and/or antibody variants or derivatives of the secondary array.

56. The method according to any one of claims 29 to 55, wherein the biological samples are derived from healthy and diseased states of a cell, tissue, organ or organism.

57. An array for use in determining the protein profile of a cell, tissue, organ or organism or a biological sample derived therefrom, comprising:

(i) a primary array of proteins $a^1_{(Xn,Yn)}$, $a^2_{(Xn,Yn)}$, $a^3_{(Xn,Yn)}$,.....,$a^n_{(Xn,Yn)}$ comprising a multiplicity of protein elements designed to emulate a significant portion of the antigenic diversity of a cell, tissue, organ, organism from which the biological sample is derived, wherein a^1,a^2,a^3,....,a^n are proteins; Xn is the coordinate of any particular protein along a first dimension of said array; Yn is the coordinate of any particular protein along a second dimension of said array; and wherein n is any positive finite number; and

(ii) a secondary array of monoclonal antibodies and/or antibody variants or

derivatives $Ab^1_{(Xn,Yn)}$, $Ab^2_{(Xn,Yn)}$, $Ab^3_{(Xn,Yn)}$, ..., $Ab^n_{(Xn,Yn)}$, wherein $Ab^1, Ab^2, Ab^3, \ldots, Ab^n$ are monoclonal antibodies and/or antibody variants or derivatives that bind specifically or non-specifically to one or more proteins in the primary array; Xn is the coordinate of any particular monoclonal antibody and/or antibody variant or derivative along a first dimension of said array; Yn is the coordinate of any particular monoclonal antibody and/or antibody variant or derivative along a second dimension of said array; and wherein n is any positive finite number.

58. An array of monoclonal antibodies or antibody variants or derivatives comprising $Ab^1_{(Xn,Yn)}$, $Ab^2_{(Xn,Yn)}$, $Ab^3_{(Xn,Yn)}$, ..., $Ab^n_{(Xn,Yn)}$, wherein $Ab^1, Ab^2, Ab^3, \ldots, Ab^n$ are monoclonal antibodies and/or antibody variants or derivatives, Xn is the coordinate of any particular monoclonal antibody and/or antibody variant or derivative along a first dimension of said array, Yn is the coordinate of any particular monoclonal antibody and/or antibody variant or derivative along a second dimension of said array, n is any positive finite number; and wherein said array is produced by a method comprising:

(i) preparing a primary array of proteins $a^1_{(Xn,Yn)}$, $a^2_{(Xn,Yn)}$, $a^3_{(Xn,Yn)}, \ldots, a^n_{(Xn,Yn)}$ comprising a multiplicity of protein elements designed to emulate a significant portion of the antigenic diversity of a cell, tissue, organ, organism from which the biological sample is derived, wherein $a^1, a^2, a^3, \ldots, a^n$ are proteins; Xn is the coordinate of any particular protein along a first dimension of said array; Yn is the coordinate of any particular protein along a second dimension of said array; and wherein n is any positive finite number;

(ii) screening the primary array with a plurality of monoclonal antibodies and/or antibody variants and/or derivatives one-at-a-time, or a reduced pool thereof one-at-a-time, so as to determine those antibodies and/or antibody variants or derivatives that bind to one or more proteins in said primary array;

(iii) preparing said secondary array of monoclonal antibodies and/or antibody variants or derivatives using those monoclonal antibodies and/or antibody variants or derivatives that bind specifically or non-specifically to one or more

proteins in the primary array.

59. The array according to claims 57 or 58, wherein the primary array comprises elements derived from one or more complex mixtures of proteins resolved by two-dimensional gel electrophoresis.

60. The array according to any one of claims 57 to 59, wherein the primary array and/or the secondary array are/is bound to a solid porous or non-porous support or matrix.

61. The array according to claim 60, wherein the solid porous or non-porous support or matrix comprises a polyvinylidene difluoride (PVDF) membrane, nitrocellulose membrane, nylon membrane or other porous or non-porous membrane to which the proteins of the primary array and/or the monoclonal antibodies and/or antibody variants or derivatives of the secondary array are transferred.

62. The array according to claim 60, wherein the solid support or matrix comprises a plurality of polymeric pins.

63. The array according to claim 60, wherein the solid support or matrix comprises a plurality of microtitre wells.

64. The array according to claim 60, wherein the solid support or matrix comprises one or more silicon chips.

65. The array according to any one of claims 57 to 64, wherein one or more of the monoclonal antibodies and/or antibody variants or derivatives of said array are derived from hybridomas or other cells which each express antibodies or antibody variants or derivatives which bind to one or more proteins in the primary array.

66. The array according to any one of claims 57 to 64, wherein one or more of the antibody variants or derivatives of said array are derived from bacteriophage or virus particles which each express antibodies or antibody variants or derivatives that bind to one or more proteins in said primary array.

67. The array according to any one of claims 57 to 66, wherein the proteins of the primary array are synthetic peptides, synthetic oligopeptides or synthetic polypeptides.

68. The array according to any one of claims 57 to 66, wherein the proteins of the primary array are recombinant peptides, recombinant oligopeptides or recombinant polypeptides.

69. The array according to claims 67 or 68, wherein the synthetic or recombinant peptides, oligopeptides or polypeptides are derived from one or more peptide libraries, and/or induced peptide expression libraries and/or protein expressed from within one or more cloned gene libraries.

70. The array according to any one of claims 57 to 66, wherein the proteins of the primary array are naturally-occurring peptides, oligopeptides, polypeptides, proteins or enzymes.

71. The array according to any one of claims 57 to 70, wherein one or more of the monoclonal antibodies or antibody variants or derivatives is labelled with one or more reporter molecules.

72. The array according to claim 71, wherein the reporter molecule is an immunogenic peptide or protein region, a FLAG peptide, poly-His amino acid sequence or poly-Lys amino acid sequence or other known amino acid string, protein A, lectin, a secondary antibody, isotope, fluorescent or enzymatic tag.

73. A method of diagnosing a human or animal subject for a medical condition, ailment, illness or immune response or a predisposition for said medical condition, ailment or illness, said method comprising:

(i) screening the array according to any one of claims 57 to 72 with a biological sample derived from said subject comprising a cell, tissue, or organ sample, bodily fluid sample, blood or serum sample, or a fraction, derivative or protein extract of any one or more of said samples; and

(ii) comparing the proteins detected for the biological sample at (i) with the proteins detected for a biological standard derived from a healthy individual, wherein differences between the biological sample the biological standard are indicative of said medical condition, ailment, illness or predisposition.

74. The method according to claim 73 further comprising obtaining the biological sample from the subject prior to screening.

75. The method according to claims 73 or 74, further comprising preparing the array for screening with the biological sample.

76. The method according to claim 75, wherein the array is prepared by selecting monoclonal antibodies or antibody variants that bind to proteins in a primary array which are derived from a healthy individual and from the same cell-type, tissue-type, organ-type, bodily fluid-type, blood-type, or serum-type as the biological sample.

77. A method of diagnosing a human or animal subject for a medical condition, ailment, illness or immune response or a predisposition for said medical condition, ailment or illness, said method comprising:

(i) separately screening either or both the primary and/or secondary arrays of the array according to any one of claims 57 to 72 with:

(a) a biological sample derived from said subject comprising a cell, tissue, or organ sample, bodily fluid sample, blood or serum sample, or

a fraction, derivative or protein extract of any one or more of said samples; and

(b) a biological standard derived from a healthy individual; and

(ii) comparing the proteins detected for said biological sample with the proteins detected for said biological standard at (i), wherein differences between the biological sample the biological standard are indicative of said medical condition, ailment, illness or predisposition.

78. The method according to claim 77 wherein the biological sample and the biological standard are derived from the same cell-type, tissue-type, organ-type, bodily fluid-type, blood-type, or serum-type.

79. The method according to claims 77 or 78 further comprising obtaining the biological sample from the subject prior to screening.

80. The method according to any one of claims 77 to 79, further comprising preparing the array for screening with the biological sample and the biological standard.

81. The method according to claim 80, wherein the array is prepared by selecting monoclonal antibodies or antibody variants that bind to proteins in a primary array which are derived from a healthy individual and from the same cell-type, tissue-type, organ-type, bodily fluid-type, blood-type, or serum-type as the biological sample.

82. The method according to any one of claims 77 to 81, for diagnosing an immune response in a human or animal subject, wherein the biological sample comprises blood or serum or a fraction, derivative or protein extract thereof.

83. The method according to claim 82, wherein the biological standard comprises blood or serum or a fraction, derivative or protein extract thereof.

84. A composition for the therapeutic or prophylactic treatment of a human or other animal subject comprising a suite of protein elements and/or responsive antibody elements of relevance to disease genesis and/or disease susceptibility that have been identified by screening the array according to any one of claims 57 to 72 in combination with a pharmaceutically-acceptable carrier or diluent.

85. The composition according to claim 84, where said composition elicits or stimulates an immune response in the subject when administered thereto.

86. The composition according to claim 85, wherein the immune response is a protective cellular and/or humoral immune antibody response.

87. A method of therapeutic treatment of a human or animal subject for a medical condition, ailment, or illness comprising administering the composition according to any one of claims 84 to 86 to said subject for a time and under conditions sufficient for the symptoms of said medical condition, ailment, or illness to abate.

88. A method of prophylactic treatment of a human or animal subject for a predisposition to a medical condition, ailment, or illness comprising administering the composition according to any one of claims 84 to 86 to said subject for a time and under conditions sufficient for an antibody response or protective immune response to occur.

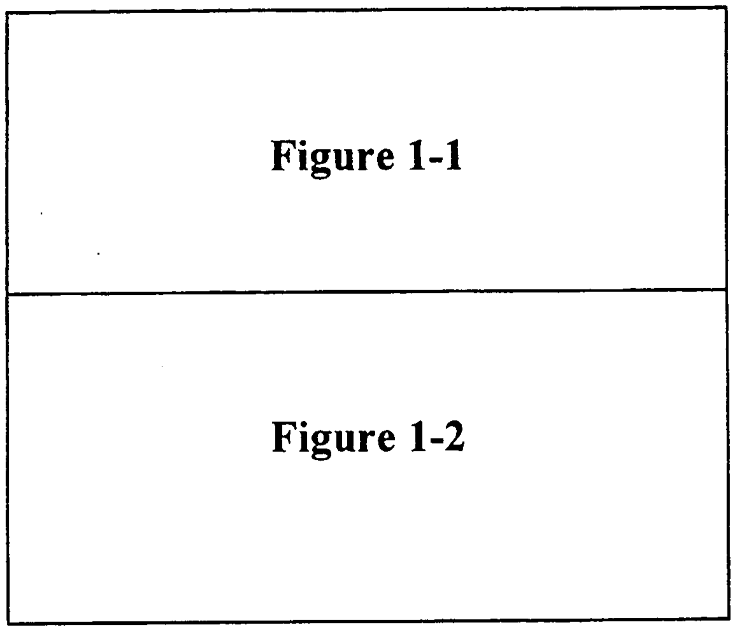


FIGURE 1

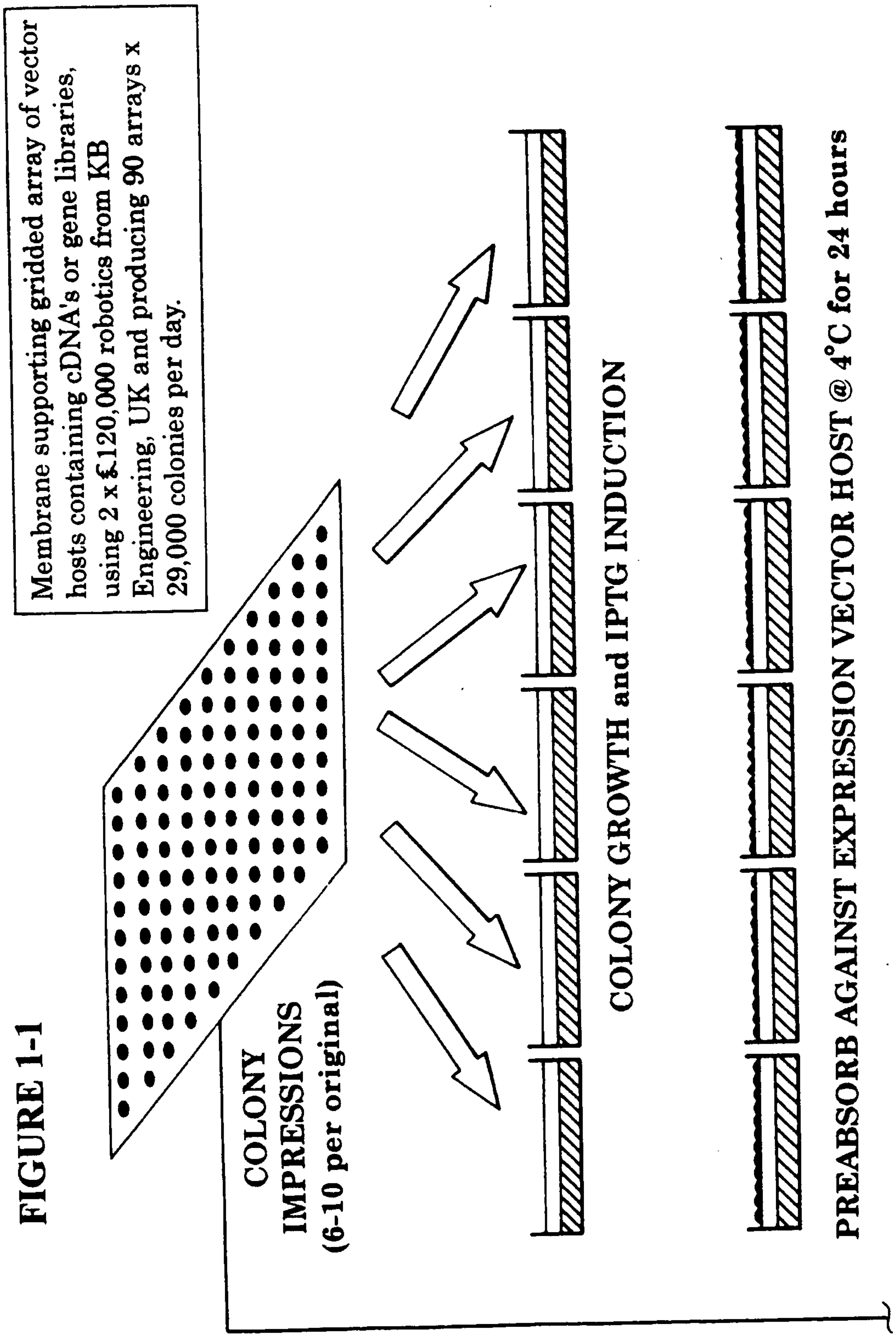

Membrane supporting gridded array of vector hosts containing cDNA's or gene libraries, using 2 x £120,000 robotics from KB Engineering, UK and producing 90 arrays x 29,000 colonies per day.
COLONY IMPRESSIONS (6-10 per original)
COLONY GROWTH and IPTG INDUCTION
PREABSORB AGAINST EXPRESSION VECTOR HOST @ 4°C for 24 hours

FIGURE 1-1

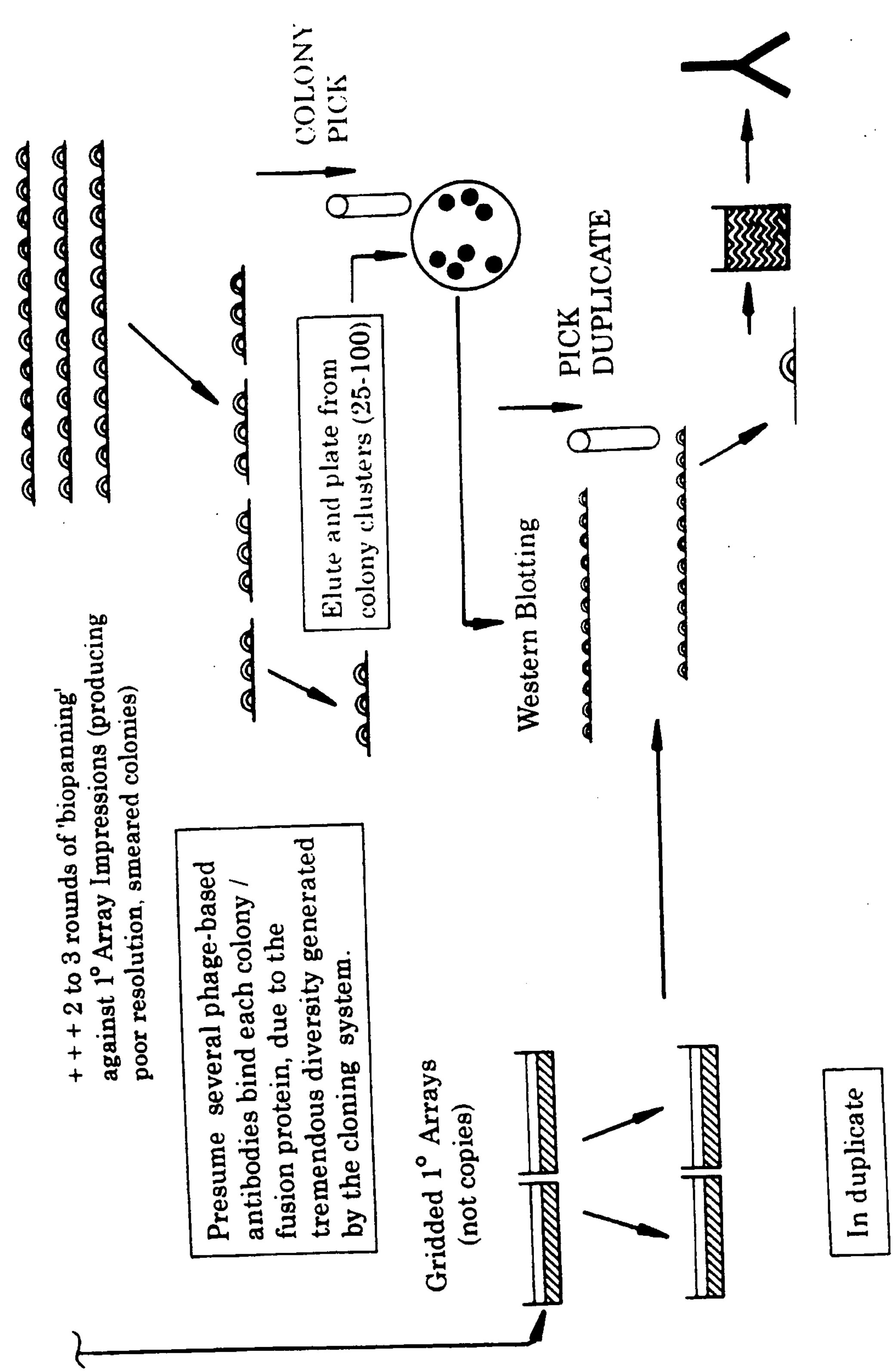

+ + + 2 to 3 rounds of 'biopanning' against 1° Array Impressions (producing poor resolution, smeared colonies)
Presume several phage-based antibodies bind each colony / fusion protein, due to the tremendous diversity generated by the cloning system.
Gridded 1° Arrays (not copies)
In duplicate
Elute and plate from colony clusters (25-100)
COLONY PICK
Western Blotting
PICK DUPLICATE

FIGURE 1-2

B-cell Numbers

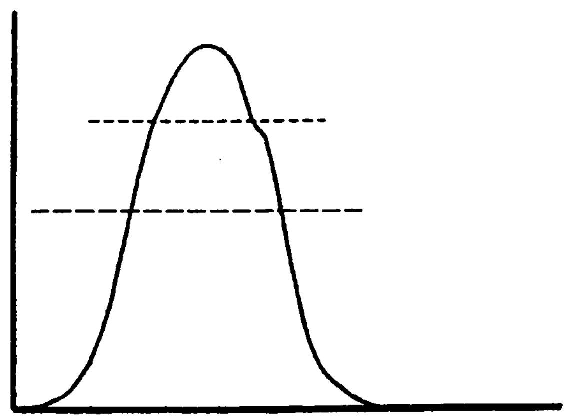


FIGURE 2

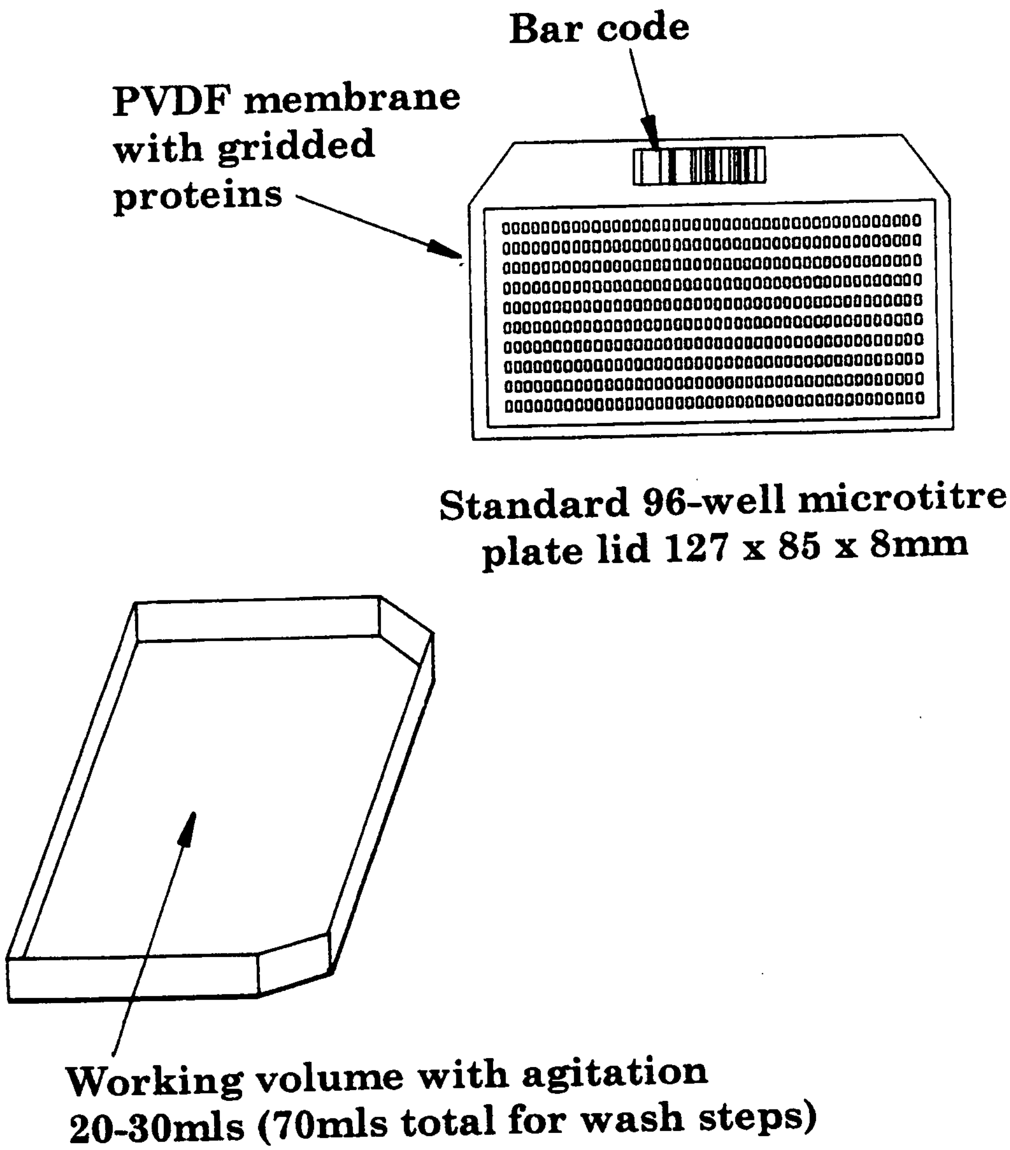
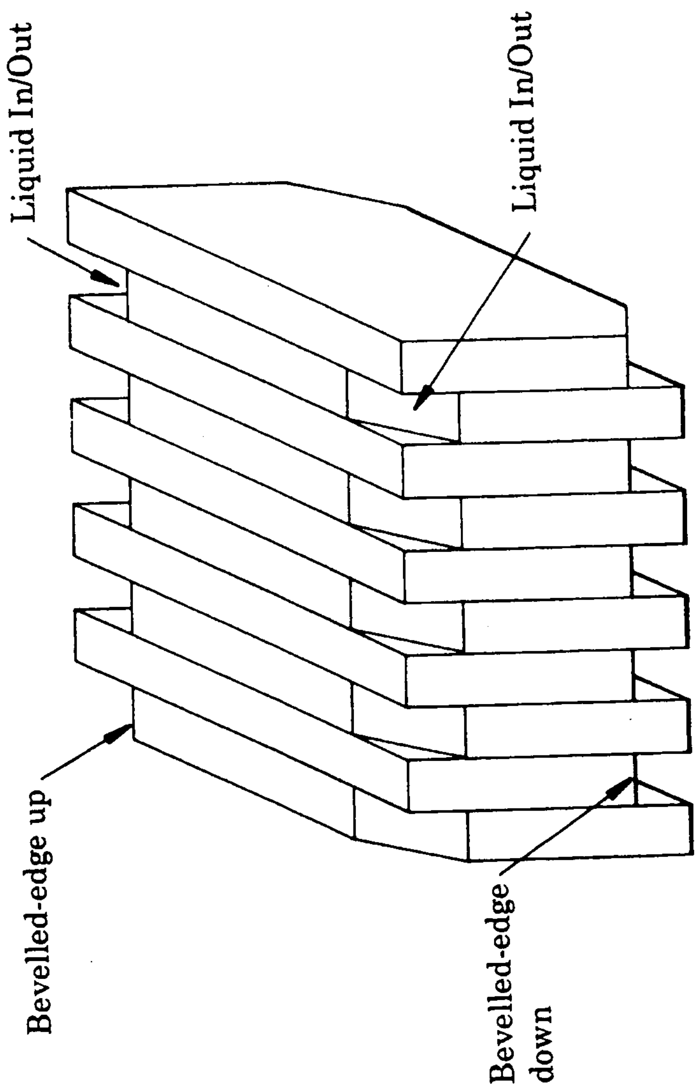

Bar code
PVDF membrane with gridded proteins
Standard 96-well microtitre plate lid 127 x 85 x 8mm
Working volume with agitation 20-30mls (70mls total for wash steps)

FIGURE 3-1

FIGURE 3-2



A stack of 50 lids is capable of holding 50 x 30,000 antigens or antibodies during high throughput Western Blotting and occupies just 127 x 85 x 435mm

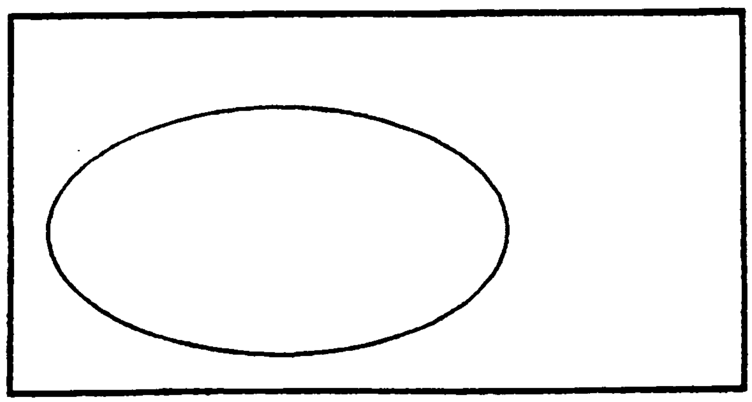


FIGURE 4

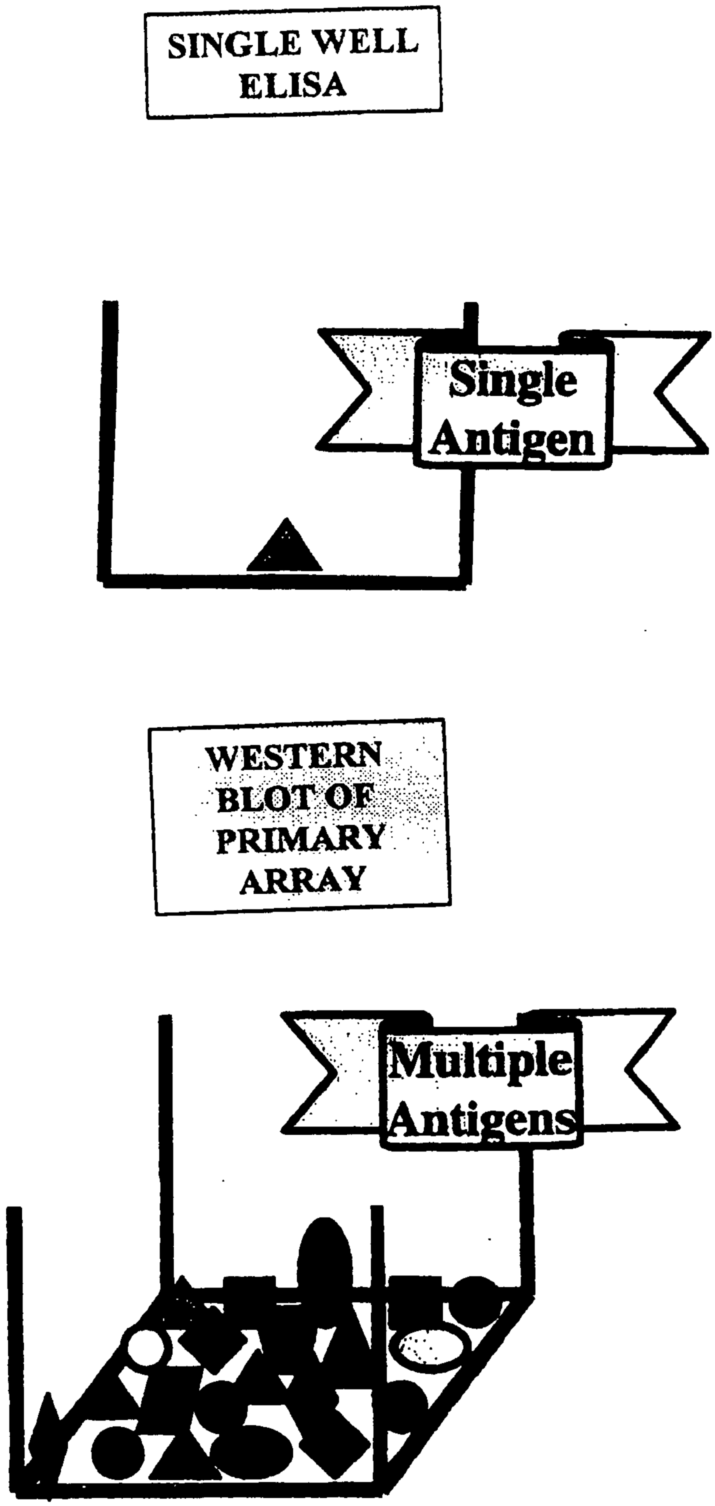

SINGLE WELL ELISA
Single Antigen
WESTERN BLOT OF PRIMARY ARRAY
Multiple Antigens

FIGURE 5

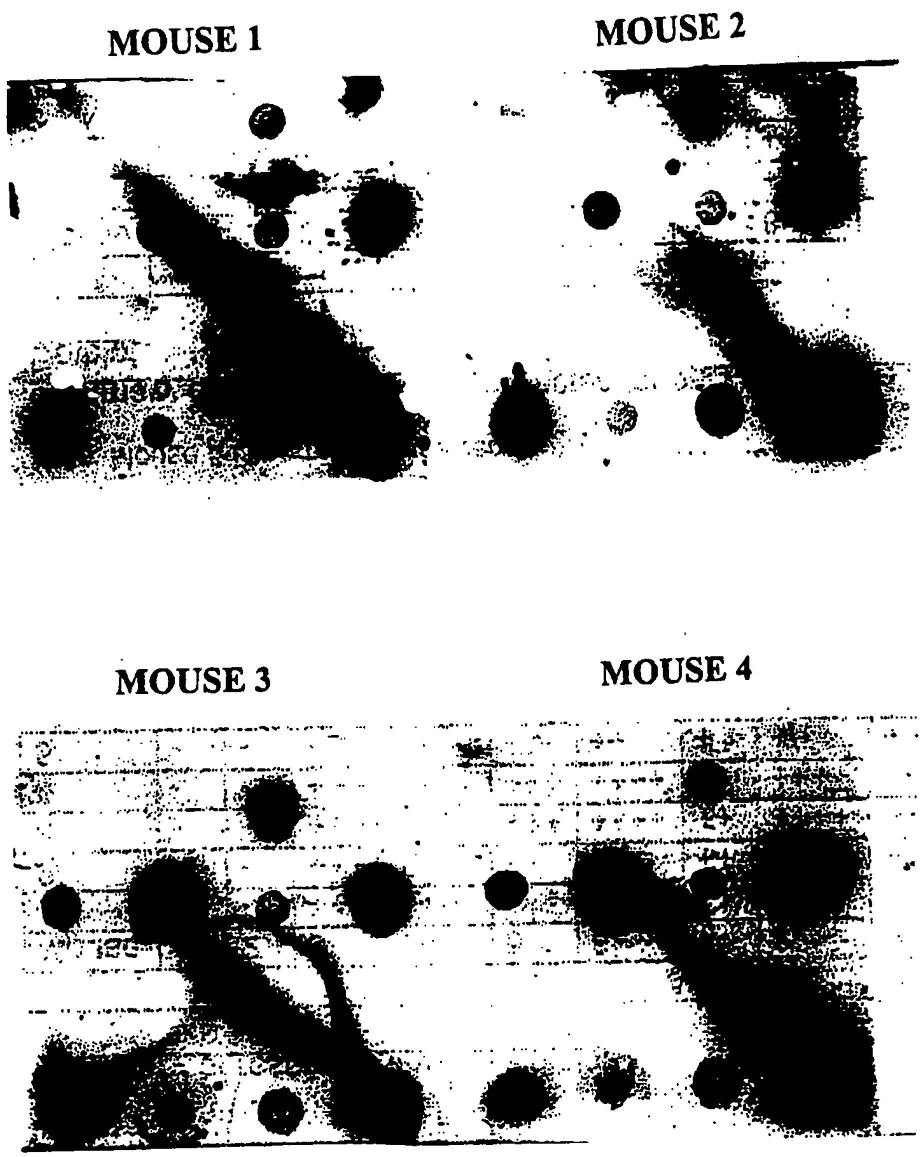


FIGURE 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00060

A.	**CLASSIFICATION OF SUBJECT MATTER**
Int Cl^6:	G01N 33/68, 33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC^6 G01N 33/ , H01J/ (searched via Derwent database)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See database search.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See attachment page.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X.Y	WO 84/03151 A1 (Centocor, Inc.) 16 August 1984; see pages 2-3 in particular.	1-88
X,Y	EP 0063810 A1 (Ciba Geigy AG) 3 November 1982; see the examples in particular.	1-88
P,X,Y	US 5763158 (Bohannon) 9 June 1998; see the claims in particular.	1-88

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

[See attached request]

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 April 1999	15 APR 1999

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,Y	EP 0818467 A2 (NES CORPORATION) 11 July 1997; see whole document.	1-88
X,Y	WO 96/30761 A1 (WARNER-LAMBERT COMPANY) 3 October 1996; see whole document.	1-88
X,Y	GB 2266182 A (Sharp Kabushiki Kaisha) 20 October 1993; see whole document.	1-88
P,X,Y	WO 98/49557 A1 (B-E SAFE, INC.) 5 November 1998; see whole document.	1-88
X,Y	Aizawa, M. et al. (1995) Integrated molecular systems for biosensors, Sensors and Actuators, vol. B 24-25, 1-5; see whole document.	1-88
X,Y	Aizawa, M. et al. (1996) Molecular assembly technology for biosensors, Nanofabrication and Biosystems: integrated materials science, engineering and biology, 222-233; see whole document.	1-88

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

1. DERWENT DATABASES.

SS 1: PROTE: (577838)
WPAT (422218) JAPIO (155620)
SS 2: ANTIBOD: (35196)
WPAT (28094) JAPIO (7102)
SS 3: ARRAY: (135546)
WPAT (87388) JAPIO (48158)
SS 4: G01N-033/IC OR H01J-049/IC (96453)
WPAT (67570) JAPIO (28883)
SS 5: 1 or 2 (599184)
WPAT (438031) JAPIO (161153)
SS 6: 5 AND 3 (3747)
WPAT (2766) JAPIO (981)
SS 7: 4 (171)
WPAT (141)

2. STN EXPRESS DATABASES

FILE 'CA'
E PROTEIN/CT
E E5+ALL

FILE 'MEDLINE'
E PROTEIN/CT
E VECTOR/CT
E GENETIC VECTORS/CT
E E3+ALL

L1	91	S PROTEOME
L2	16011	S ARRAY
L3	1	S L1 AND L2
L4	10412811	S PROTEIN
L5	511300	S ANTIBODY
L6	1400906	S L1 OR L4 OR L5
L7	4578	S L6 AND L2
L8	197	S L6 (1N) L2
		E ANALYST/CT
L9	46	S E3 AND L8
L10	42	S L9 NOT (DNA OR RNA)

FILE 'CA'

L11	11233117	S PROTEIN
L5	257317	S ANTIBODY
L6	0	S L10
L7	0	S L9
L8	110	S L8 AND ANALYSIS
	94	E L15 NOT (DNA OR RNA)

3. IP Australia database search for applicants; Ciphergen Biosystems, Inc. (related art); Hutchens and Yip (related art).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU 99/00060

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	8403151	EP	135541	US	4591570	WO	8403151
EP	63810	AR	231590	AU	83069/82	BR	8202492
		CA	1200761	EP	63810	ES	511735
		ES	8400199	ES	523722	ES	8405156
		ES	8405157	FI	821441	GB	2099578
		GR	75430	HK	538/88	IL	65627
		JP	58009070	MX	160043	NO	821411
		NZ	200443	PH	26773	PT	74816
		SG	252/88	US	5486452	ZA	8202896
EP	818467	JP	10025299				
WO	9630761	AU	47702/96				
GB	2266182	JP	6163876				
WO	9849557	AU	71649/98				

END OF ANNEX